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Ancient habitat shifts and organismal diversification are decoupled in the African viper genus *Bitis* (Serpentes: Viperidae).

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Running Title: Diversification in vipers (*Bitis*)

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ABSTRACT

Aim The expansion of open habitats during the mid-Miocene has been hypothesised as a driver of allopatric speciation for many African taxa. This habitat-dependent mode of diversification has been implicated in the shift from C_3 (e.g. forest/woodland) to C_4 dominated systems (i.e. open savanna, grasslands) in a number of African squamates. We examined this hypothesis using a genus of African viperid snakes (*Bitis*) with both open habitat and forest-dwelling representatives.

Location Africa.

Methods A comprehensive multilocus dataset was used to generate a calibrated species tree using a multispecies coalescent model. Individual gene trees and patterns of nuclear allele sharing were used to assess species monophyly and isolation. To test the habitat-dependent evolution hypothesis, we generated an ancestral character state reconstruction for open and closed habitats using the dated phylogeny. This was related to the timing of open habitat expansion and forest/woodland contraction in Africa.

Results The genus *Bitis* originated in the Oligocene, with species level diversification in the late Miocene/Pliocene. Four well-supported clades correspond to the recognised subgenera *Bitis, Keniabitis, Macrocerastes* and *Calechidna*. Several previously unrecognised lineages potentially represent cryptic species.

Main Conclusions Habitat-dependent evolution does not appear to have been a main driver for generic level viperine diversification: the ancestral state for *Bitis* was open habitat and at least one clade moved into forest in the Miocene, long after forest had contracted and fragmented. Forest dependent species diversified only in the late Miocene, presumably as forest became further reduced in extent, fitting an allopatric model of speciation. Although our results do not favour a general pattern of habitat-dependent diversification in *Bitis*, cladogenesis within the subgenus *Calechidna* for 'arenicolous' species (*B. caudalis* complex) and 'rupicolous' species (*B. atropos-cornuta* complex), corresponds to the aridification of southwest Africa. This suggests there are subtleties not captured in the broad open habitat category, which are relevant for understanding the role of habitat-dependent evolution.

Keywords
sub-Saharan Africa, multilocus phylogenetics, multispecies coalescent, reptiles, snakes

INTRODUCTION

In broad terms, sub-Saharan African faunal lineages can be segregated into those that occupy closed or dense canopy forest/woodland ecosystems ("forest" lineages) and those that occupy structurally more open ecosystems such as grassland, heathlands, open savanna and desert ("open-habitat" lineages: e.g. deMenocal, 1995, 2004; Tolley *et al*., 2008, 2011; Maslin *et al*., 2014). Through the Paleogene (66-23 Mya) dense woodland/forest were widespread across sub-Saharan Africa, and were gradually displaced by open ecosystems through the Oligocene and early Miocene as the tropical climate aridified (Coetzee, 1993; Morley, 2007; Kissling *et al*., 2012; Linder 2017). During the Oligocene, forest/woodland became reduced in extent, contracting from North Africa and the Southern & Zambezian region into Central Africa presumably leaving substantial patches in central Africa (see Morley, 2007; Figs S1 & S2 in Supporting Information), possibly as a mosaic with more open vegetation types (Linder, 2017). From the mid to late Miocene, beginning ca. 10 million years ago (mya), open habitats expanded markedly, with those comprised primarily of plant species utilising the C_4 photosynthetic pathway becoming increasingly dominant on the continent (Couvreur *et al*., 2008; Edwards *et al*., 2010; Kissling *et al*., 2012; Maslin *et al*., 2014). Subsequent climatic cooling and aridification during the Pliocene and Pleistocene, 2.8–1.0 mya, was associated with further open habitat expansion and the dominance of C_4 grasslands and savanna (deMenocal, 1995; Kissling *et al*., 2012). This aridification was punctuated by short moist periods that could have facilitated temporary forest re-expansion (Trauth *et al*., 2005; Maslin *et al*., 2014). Regardless, since the Cretaceous, the widespread forest/woodland lost most of its extent, with open habitats becoming dominant in the landscape (Morley, 2007; Kissling *et al*., 2012).

The prominent expansion of open habitats in sub-Saharan Africa is thought to have played a key role in the evolution of open habitat fauna. Multiple hypotheses have been invoked to explain this faunal evolution in open habitats (Vrba, 1985, 1992; Potts, 1998), and collectively these have been termed "habitat-specific hypotheses" (Potts, 1998; deMenocal, 2004). The paradigm essentially points to ecological speciation, where diversification is driven by directional selection in differing environments (e.g. Rundil & Nosil, 2005; Schluter, 2009). Here, we adopt the term 'habitat-dependent' evolution to specifically refer to ecological diversification of lineages inhabiting novel habitats due to reorganisation of habitat types on the African continent.

The mammalian fossil record provides considerable evidence for habitat-dependent evolution in sub-Saharan Africa. In particular, the expansion of C_4 grassland during the Plio-Pleistocene appears to have played a role (Hewitt, 2004) as the first appearance of many arid adapted species across a range of taxa coincides with this period (Wesselman, 1985; Vrba, 1992; Bobe *et al*., 2002; Bobe & Behrensmeyer, 2004; Bowie & Fjeldså, 2008). Phylogenetic studies also support this hypothesis, with a number of forest dependent taxa showing strong signatures of allopatric speciation corresponding to fragmentation of forests (Bowie *et al*., 2006; Tolley *et al*., 2008; Lawson, 2010; Demos *et al*., 2014; Menegon *et al*., 2014; Barej *et al*., 2015), whereas recent radiations appear to correspond with occupation of more open habitats (Tolley *et al*., 2006; Bowie & Fjeldså, 2008; Tolley *et al*., 2013; Demos *et al*., 2014). These patterns are clearly taxon dependent, presumably because of the idiosyncratic life-history characteristics and dispersal ability of the taxa. In general however, highly vagile species are either generalists, or can disperse across unsuitable habitat (Oatley

et al., 2012; Fuchs *et al*., 2013), which facilitates gene flow resulting in low genetic structure. In contrast, most forest dependent species will find the open habitat a formidable barrier and require either forest reconnection or habitat corridors to maintain population connectivity and gene flow (Bowie *et al*., 2006; Measey & Tolley, 2011; Barej *et al.*, 2015; Bittencourt-Silva *et al*., 2016). Given taxon idiosyncrasies, a universal model for the evolution of fauna on the continent is not plausible. However, a paradigm that incorporates the reduction of forest/woodland as an important driver of biogeographic patterns is tenable and can incorporate the idiosyncratic nature of species.

Squamate reptiles are a taxonomic group that is both widespread and highly diverse within sub-Saharan Africa, where diversification of forest/woodland dependent taxa has been influenced by habitat shifts. For example, several clades of squamates that currently occupy open habitats diversified within the Miocene (e.g. chameleons and snakes; Wüster *et al*., 2007; Pook *et al*., 2009; Barlow *et al.*, 2013; Tolley *et al.*, 2013). Furthermore, ancient forest lineages in the southern African chameleon genus *Bradypodion* gave rise to open-habitat species following the onset of open habitat expansion in the Pliocene (Tolley *et al*., 2008; Measey *et al*., 2009; Edwards *et al*., 2012; da Silva *et al*., 2014; da Silva & Tolley, 2017), suggesting that shifts to open habitats beginning in the Miocene may have been widespread on the landscape and across multiple taxonomic groups.

The African viper genus *Bitis* provides an opportunity to test the habitat-dependent hypothesis of ecological diversification. Commonly referred to as the African adders, *Bitis* is Africa's most taxonomically diverse and geographically widespread viperid genus, containing eighteen extant species (*sensu* Lenk *et al*., 1999; Branch, 1999, Gower *et al*., 2016; Uetz *et al*., 2017) and one documented extinct Pleistocene species, *Bitis olduvaiensis* (Rage, 1973). Several studies have investigated the phylogeny of *Bitis* using morphological evidence (Groombridge, 1980; Ashe & Marx, 1988; Wittenberg *et al*., 2015) and immunological distances (Lenk *et al.*, 1999). Higher level phylogenies of Viperidae and Viperinae have also included *Bitis* (Herrmann & Joger, 1995, 1997; Herrmann *et al*., 1999; Lenk *et al.*, 1999, 2001; Wüster *et al.*, 2008; Pyron *et al.*, 2013; Alencar *et al.*, 2016). The study of Lenk *et al.* (1999) identified four major mitochondrial clades within the genus *Bitis*, which were formally recognised as subgenera (Table 1). These are:

- *Macrocerastes*, a clade of large-bodied forest adders, which includes the Gaboon adders (*B. gabonica* and *B. rhinoceros*) and the Rhinoceros viper (*B. nasicornis*).
- *Calechidna*, a clade of open habitat dwarf adders endemic to southern Africa. This clade is further divided into two subclades corresponding, respectively, to those taxa primarily associated with gravel or rocky habitats ("rupicolous", *B. atropos-cornuta* complex) and those associated with sandy substrates ("arenicolous", *B. caudalis* complex).
- *Keniabitis*, a monotypic clade representing the small-bodied Kenyan endemic *B. worthingtoni*, which occurs in montane grassland habitats along the Kenyan Rift Valley.
- *Bitis* (the type subgenus), representing the geographically widespread and large-bodied puff adder (*B. arietans*), which occurs across a variety of open woodland, grassland and scrubland habitats throughout sub-Saharan Africa, southern Arabia and Morocco.

Although the evolutionary relationships within *Bitis* are relatively well understood, several important questions remain. The relationship between the subgenera lacks resolution, and the phylogenetic positions of *B. (K.) worthingtoni* and *B. (B.) arietans* were equivocal in

previous analyses due to a lack of statistical support at basal nodes (Lenk *et al*., 1999; Wüster *et al*., 2008; Pyron *et al*., 2013). In addition, several poorly known species have not been included in any molecular phylogeny to date (*B. harenna*, *B. albanica*, *B. heraldica* and *B. inornata*), and most studies of *Bitis* have utilised single individuals to represent species, precluding any assessment of levels of intraspecific genetic diversity or the testing of species monophyly (but see Barlow *et al*., 2013).

In this study, we examine evolutionary relationships within *Bitis* to investigate whether a habitat-dependent hypothesis of diversification applies to this genus. We used a time-calibrated multilocus phylogeny, including 16 of the 18 currently recognised *Bitis* species, to explore patterns and timing of diversification among the subgeneric clades. In particular, we expected that *Bitis* lineages occupying open habitats (subgenera: *Calechidna*, *Keniabitis* and *Bitis*) diverged either in response to the initial but gradual aridification of Africa (Eocene/Oligocene) or later, during the rapid mid-Miocene expansion of open habitats. If so, the origin of the genus should reflect the geographic region where the forest/woodland contraction was maximal during those time periods (either North Africa or the Southern & Zambezian regions). We carried out ancestral character state reconstruction of the broad habitat categories (forest/woodland mosaic and open-habitat), to understand if the timing of diversification corresponded to major habitat shifts on the continent, which could support habitat-dependent diversification. Furthermore, an ancestral area reconstruction allowed us to assess whether the geographic origin of key clades fits well with habitat-dependent diversification. We also included multiple representatives of species to investigate the outstanding taxonomic issues, specifically subgeneric and species monophyly and the possibility of cryptic speciation.

MATERIAL AND METHODS

Tissues (scale clips, blood, shed skins, dermal tissue or liver) were sampled from all currently recognised *Bitis* species except the poorly known Angolan species *B. heraldica* and the recently described *B. harenna*. All individuals were released after sampling or retained alive by their owners. Multiple representatives of each sampled species were included except for *B. inornata* and *B. rhinoceros*, for which it was only possible to sample a single individual. Sequences from additional representatives of the Viperidae were also generated or downloaded from GenBank for use as outgroup taxa and to facilitate the dating analysis. Outgroup taxa included one to three individuals from six other genera (from Africa and Eurasia) in the subfamily Viperinae, resulting in a dataset of 77 individuals for 4 genes. Of these, sequences of one to three genes from 15 individuals were available on GenBank. Details of samples, vouchers and GenBank accession numbers are given in Table S1 in Supporting Information.

We generated sequence data from two mitochondrial and two unlinked nuclear markers. The mitochondrial data consisted of partial sequences of the 16s ribosomal RNA (16s) and NADH dehydrogenase subunit 2 (ND2) genes. The nuclear markers were exonic sequences of the prolactin receptor (PRLR) and ubinuclein 1 (UBN1) genes. Total DNA was extracted from tissue samples using a Qiagen DNeasy™ Tissue Kit (cat. no. 69506) following the manufacturer's instructions. Genetic markers were PCR amplified using the following primers. 16s: L2510 (5'-CGCCTGTTTATCAAAAACAT-3') and H3080 (5'-

CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al*., 1991); ND2: L4437b (5'-CAGCTAAAAAAGCTATCGGGCCCATAC-3') (Kumazawa *et al.,* 1996) and tRNA-trpR (5'-GGCTTTGAAGGCTMCTAGTTT-3') (Ashton & de Queiroz, 2001); PRLR: PRLR-f1 (5'-GACARYGARGACCAGCAACTRATGCC-3') and PRLR-r3 (5'-GACYTTGTGRACTTCYACRTAATCCAT-3') (Townsend *et al*., 2008); UBN1: BaUBN_F (5'-CCTCTGGTTACTCAGCAGCA-3') and BaUBN_R (5'-ATTGGCCACTCCTTGTGTTC-3'). PCRs comprised 9.6 µl ABgene ReddyMix™ PCR Master Mix (cat. no. AB-0575/LD/A), 0.27 µM of each primer and 5–10 ng of template DNA, giving a final reaction volume of 11 µl. The thermocycling regimes involved an initial denaturation at 94°C for 2 min; 30–40 cycles of: 30 s denaturation at 94°C, 30 s (16s, ND2) or 60 s (PRLR, UBN1) annealing at 50°C (16s), 52°C (PRLR), 55°C (ND2), or 60°C (UBN1), and 45 s (16s, PRLR, UBN1) or 90 s (ND2) extension at 72°C; and a final extension for 5 min at 72°C. PCR products were cleaned using the enzymes exonuclease 1 and thermo-sensitive alkaline phosphatase, and direct sequencing carried out by Macrogen Inc. (dna.macrogen.com) using forward PCR primers (16s, some PRLR) or both forward and reverse PCR primers (ND2, UBN1, some PRLR).

Sequences were proof-read and aligned using the software CODONCODE ALIGNER 3.5.6 (www.codoncode.com). Only clean sequences were retained, and we re-sequenced any sequence with questionable stretches. Protein-coding gene sequences were translated to check that no frameshift mutations or stop codons were present. Alignment was ambiguous for some sections of the 16s alignment so these regions were excluded from analyses. UBN1 contained a 'TCC' tri-nucleotide repeat section with several heterozygous indels necessitating the exclusion of 30 bp.

Heterozygous positions were identified in nuclear sequence chromatograms by a combination of visual inspection for double peaks and typically low quality Phred scores (Ewing & Green, 1998) for the bases surrounding a heterozygous position. Individual allele sequences were estimated from the diploid nuclear sequences using PHASE (Stephens *et al.,* 2001; Stephens & Scheet, 2005) in DnaSP v. 5 (Librado & Rozas, 2009), using default settings. To verify the reliability of the PHASE, analysis we computed maximum likelihood trees under the GTRCAT model in RAxML 7.2.8 (Stamatakis, 2006) for both the unphased and phased alignments, with clade support assessed using 100 bootstrap replicates and specifying the *Causus* sequences as outgroup. For each nuclear gene, both phased and unphased alignments produced highly congruent topologies with broadly comparable bootstrap values for all nodes above the species level (Supporting Information Figs S3-6). Overall, this indicates no obvious distortion of phylogenetic signal in either dataset as a result of the phasing procedure. The final dataset consisted of 2415 base pairs: 16S-426bp; ND2-1014bp; PRLR-525bp; UBN1-450bp.

Species relationships were first investigated by concatenating data from all loci. A maximum likelihood (ML) search was run using RAxML HPC 7.2.8 (Stamatakis, 2006) on the CIPRES Science Gateway (Miller et al. 2010) for the 4-gene dataset. The analysis was run using both unphased and phased nuclear sequences. Each gene was partitioned separately, and the default GTR+I+G model was used with rapid bootstrapping halted automatically (Stamatakis et al. 2008). This analysis was run three times to ensure that independent ML searches produced the same topologies. We considered nodes with a bootstrap value of >70% as supported in this analysis.

The *Bitis* species tree was then inferred using a multispecies coalescent (MSC) model using *BEAST (Heled & Drummond, 2010), implemented in BEAST v. 1.7.4 (Drummond *et al*., 2012). *BEAST co-estimates individual gene trees and the species tree within which they evolved, using a fully Bayesian framework accounting for incomplete lineage sorting. We assigned individuals to species according to current taxonomy (Lenk *et al.*, 1999) except in the case of *B. caudalis*, which preliminary analysis found to comprise two polyphyletic mitochondrial lineages (see Results). Individuals corresponding to these mitochondrial lineages were therefore assigned as separate taxa (*B. caudalis* L1 and L2). Including outgroup taxa, the resulting species tree contained 24 species/taxa, sampling 77 individuals, and was inferred from three independent gene trees: mitochondrial (estimated from concatenated 16s and ND2 sequences), PRLR and UBN1.

We estimated timing of divergence among *Bitis* species by calibrating the MSC species tree analysis based on fossil evidence from the related Eurasian viperine clade (represented by *Vipera berus*, *Daboia siamensis* and *Montivipera xanthina*), which the fossil record shows to have existed at least 20 mya (Szyndlar & Rage, 1999). Based on the assumption that the most recent common ancestor (MRCA) of this clade is unlikely to have occurred considerably earlier than this, we constrained the monophyly of this clade and applied a lognormal prior to the age of the MRCA with a 20 mya offset, mean of zero and standard deviation of 1.0, and upper limit of 40 mya. Head *et al.* (2016) argued that while fossil vertebrae of the "*aspis* complex" of Szyndlar & Rage (1999) can be assigned to that lineage, other viperine vertebrae would be difficult to assign to any particular group of viperines, or even to distinguish from crotaline remains. They therefore suggested that this calibration point can only be used to date the divergence of viperines and crotalines. However, if the "*aspis* complex" fossils of Szyndlar & Rage (1999) can indeed be assigned to the genus *Vipera* based on apomorphies, then it logically follows that they can and should be used to calibrate the divergence of that genus from its sister group, most likely *Daboia* (Wüster *et al*., 2008; Pyron *et al.*, 2013; Alencar *et al*., 2016), not the older split between viperines and crotalines. Given the relative scarcity of early Miocene/Oligocene viperid fossils, we prefer a less narrowly constrained upper age limit for this calibration point than suggested by Head *et al.* (2016).

Separate, unlinked nucleotide substitution models were specified for each gene, selected from those available in BEAUTI under the Bayesian information criterion (BIC) in MEGA5 (Tamura *et al*., 2011). Uncorrelated, lognormal relaxed clock models were specified for each gene. A Yule speciation prior with piecewise linear population size model and constant root was specified for the species tree. The final analysis was carried out on Bioportal (www.bioportal.uio.no), and involved three independent runs of $5x10^8$ generations that sampled the Markov chain Monte Carlo every 50,000 generations. The first 10% of samples from each run was removed as burn in. Convergence and adequate sampling (effective sample sizes > 200) of all parameters was verified in TRACER 1.5 (Rambaut & Drummond, 2007). The maximum clade credibility tree was selected from the combined posterior sample of 27,000 species trees and annotated with posterior clade probabilities and node heights equal to the median value from the posterior sample using TREEANNOTATOR. We consider posterior probabilities ≥ 0.90 as providing moderate clade support, and those ≥0.95 as providing strong support.

We also examined the individual gene trees resulting from the *BEAST analysis, which are estimated independently of the species designations used to constrain the species tree. We checked whether current species designations correspond with monophyletic clades in the gene trees, and also looked for the existence of divergent genetic lineages within currently described species that may indicate the presence of monophyletic species complexes.

As the time taken for nuclear markers to reach reciprocal monophyly is expected to exceed that of mitochondrial markers due to an expected four-fold reduction in effective population size of the latter, we also investigated whether currently recognised species possess unique nuclear alleles. The presence of unique alleles provides evidence of lineage isolation because shared alleles are expected to be lost over time due to genetic drift, before reciprocal monophyly has been achieved. Shared alleles, in contrast, could indicate allele sharing between groups due to ongoing gene flow, or alternatively a relatively recent speciation event. The ability to detect shared alleles is governed by sample sizes, which are relatively small for the majority of species studied here. Nuclear allele sharing can thus only be seen as an additional line of evidence for lineage isolation, rather than as providing conclusive support.

As an independent indicator of relationships among subgenera, we included an additional nuclear marker, the anonymous nuclear marker Ba34 (Barlow *et al*., 2012). Ba34 sequences were not available for all species, precluding their use in the species-level *BEAST analysis. However, all four subgenera, including both sand- and rock-dwelling *Calechidna* clades, are represented by published sequences (Barlow *et al*., 2012). These were phased (as described previously) and analysed using *BEAST, assigning sequences to one of the five major *Bitis* clades. Relaxed clock models were used for data partitions and the HKY substitution model specified for Ba34. Other aspects of the analysis were as described previously.

Ancestral character state estimation for habitat was carried out using the APE 3 and PHYTOOLS packages in R (Paradis, 2012; Popescu *et al*., 2012; Revell, 2012). Each taxon was coded as occurring in closed (forest/woodland) or open (e.g. open savanna, karroid, grassland, heathland, desert) habitat (Fig. 1). Outgroup taxa were included to polarize the analysis, and were coded as belonging to open habitats (this being the dominant habitat across each outgroup genus included; Phelps, 2010). Because five Viperinae genera were missing from our analysis, we must treat the results of this analysis with caution. However, it should be noted that four of these five missing genera occur in open habitats, with only *Atheris* found in forest. A more comprehensive Viperinae phylogeny would be needed to test whether inclusion of *Atheris* and the other genera would change our results. The reconstructions were run with the 'ace' function using the equal states Markovian (Mk) model of character evolution (https://www.r-phylo.org/wiki). The ancestral habitat reconstruction analyses were also run in MESQUITE 3.6 (Maddison & Maddison, 2018) using the same character coding, a likelihood optimization, and the Mk model. Because the ML topology differed from the MSC species tree in the positon of *B. arietans* and *B. worthingtoni*, both of the ancestral habitat analyses were run on the maximum likelihood tree (pruned to retain one tip per taxon as in Fig. 1b) as well as on the MSC species tree.

An ancestral area reconstruction was carried out using a Dispersal-Extinction-Cladogenesis model (DEC; Ree & Smith, 2008) in RASP 4.0 beta (Yu *et al.*, 2015) using the ultrametric MSC species tree generated in *BEAST, and including the six outgroup genera from the Viperinae (*Causus, Cerastes, Daboia, Echis, Montivipera, Vipera*). The analysis was also run on the maximum likelihood tree (pruned to retain one tip per taxon as in Fig. 1b). The terminal taxa for *Bitis* were coded for the analysis based on their known distributions, whereas the taxa that represented the six Viperinae genera were coded according to the distribution of the entire genus (see Phelps, 2010). The following regions were used for the coding: Eurasia, North Africa (including Saharan), Sudanian, Congolian, Ethiopian, Somalian, Zambezian, Southern following the biogeographic regions from Linder *et al.* (2014; Fig. S2 & Table S2 in Supporting Information). The DEC analysis allows for both range and dispersal constraints to be defined, so that lineage dispersal can be modelled taking into account timing of divergences and the connectivity between geographic regions (Ree *et al.*, 2005). Ancestral ranges were constrained to adjoining geographic regions (Table S3 in Supporting Information). Dispersal probabilities between regions were assigned at four time points (0-2 mya, 2-11 mya, 11-30 mya, 30-47mya; Table S4 in Supporting Information) based on the potential for connectivity between regions. This was guided by present day vegetation and climate of the continent and paleo-vegetation maps for Africa (Morley, 2007; Kissling *et al.*, 2012; Fig. S1 in Supporting Information).

RESULTS

Both MSC species tree and ML analyses of the concatenated alignment supported the monophyly of *Bitis* and its subdivision into four previously recognised subgeneric clades (Fig 1, Figs S7 & S8 in Supporting Information). However, these methods supported different relationships between some major clades. The MSC species trees have *Keniabitis* (*Bitis worthingtoni*) sister to all other species of *Bitis*, and showed moderate support (0.90 pp) for *Bitis arietans* as sister to *Calechidna* + *Macrocerastes*. In contrast, the ML topology for the concatenated alignment shows *B. arietans* (100% bootstrap) as sister to all other species (Fig. 1b, Fig. S8 in Supporting Information). The topologies from the ML and MSC analyses for the four-gene dataset also differed slightly for some clades within the *Calechidna* (Fig. 1b), although the ML and mitochondrial gene tree generated in the MSC analysis were in agreement for these relationships (Fig. 2).

In other respects, topologies from the two methods (MSC and ML) were in agreement, and there were no discrepancies between the unphased (Fig. S8 in Supporting Information) and phased (figure not included) ML topologies. Furthermore, the *BEAST analysis supported monophyly of the four subgeneric clades for each individual gene tree (Figs S9-10 in Supporting Information), with the exception of *Calechidna*, for which monophyly was not supported in the PRLR and UBN1 trees. The position of *B. arietans* was sister to all other *Bitis* in the PRLR tree, albeit without notable support. The inclusion of sequences of the anonymous nuclear marker Ba34 provided improved resolution of relationships among the major clades (Fig. S11 in Supporting Information), providing strong support for the *Calechidna* + *Macrocerastes* + *B. arietans* clade (posterior probability 0.95 compared to 0.90 in the three locus analysis).

Relationships among the four representatives of the subgenus *Macrocerastes* are well resolved in the species and ML trees, with the two Gaboon adders (*B. rhinoceros* and *B.*

gabonica) sister to each other. *Bitis nasicornis* forms the sister group to this Gaboon adder clade, with *B. parviocula* in turn sister to this clade (Fig. 1). Individual gene trees largely recovered identical relationships and the monophyly of all species was strongly supported with the exception of *B. nasicornis* in the UBN1 tree (Fig. S10 in Supporting Information). All recognised species exhibited unique alleles with the exception of *B. rhinoceros* and *B. gabonica*, which share PRLR alleles (Fig. 2b).

Species tree and ML analyses supported the subdivision of *Calechidna* into two clades corresponding to the rupicolous and arenicolous dwarf adders. Most members in the rupicolous clade are within a recent radiation *(*Fig. 1; *B. albanica*, *B. armata*, *B. cornuta*, *B. inornata* and *B. rubida*). *Bitis rubida* is paraphyletic with respect to *B. albanica* in the mitochondrial gene tree, and the occurrence of shared nuclear alleles is widespread among these five taxa (Fig. 2b). Monophyly of the remaining species within the rupicolous clade was supported across all gene trees. Notably a single *B. atropos* individual from Zimbabwe is divergent from South African individuals in the mitochondrial and UBN1 gene trees and also possesses unique alleles for both nuclear markers (Fig. 2b, Fig. S10 in Supporting Information).

Within the arenicolous *Calechidna* clade, the monophyly of *B. schneideri* was strongly supported across all analyses and it does not share any nuclear alleles with other species (Fig. 2b). The monophyly of *Bitis caudalis* was not supported in any of the analyses. Furthermore, the two polyphyletic mitochondrial lineages (*B. caudalis* L1 and L2) also failed to form a monophyletic group in the species and ML trees, with an alternative sister species relationship between *B. caudalis* L2 and *B. schneideri* being moderately supported (Fig. 1). This relationship was fully supported in the mitochondrial tree, with no nuclear allele sharing (Fig. 2). Further examination of the posterior sample of species trees showed that *B. caudalis* was paraphyletic in 98.9% of the posterior sample. The monophyly of *B. peringueyi* was supported in the mitochondrial and the ML trees, and this species shares nuclear alleles with *B. caudalis* L1 (Fig. 2b).

The dating analysis using a single Eurasian viperine fossil calibration provided a median estimated age for the basal divergence of *Bitis*, and the origin of the *Keniabitis* lineage, of 26.4 mya (95% credibility interval (CI) 20.7–33.7 mya). Divergence of the *B. arietans* lineage occurred 23.5 mya (95% CI 18.1–29.5 mya), and the *Macrocerastes* and *Calechidna* lineages separated 18.9 mya (95% CI 14.6–23.7 mya). The two *Calechidna* clades are estimated to have diverged 15.2 mya (95% CI 10.0–20.0 mya). Ancestors of the extant species within *Macrocerastes* and *Calechidna* are estimated to have arisen within approximately the last 10.5 my, with the most recent speciation events occurring in the *cornuta-inornata* (rupicolous) complex, which radiated within approximately the last 0.1–1.3 my.

The ancestral habitat state for the genus is unambiguously open habitat for both the APE and MESQUITE analyses. In addition, the estimated marginal ancestral states at each node were unequivocal with all proportional likelihood values > 0.98 (Fig. 1a, Fig. S12 in Supporting Information). There is a single transition to forest in the *Macrocerastes* clade, with no transitions out of that habitat. The ancestral habitat reconstructions based on the ML topology produced essentially the same support values (> 0.98) for character states at each node (results not shown).

The ancestral area reconstruction with the DEC analysis suggests that *Bitis* originated in the Zambezian and Somalian/Ethiopian biogeographic regions (Fig. 3, Table S5 in Supporting Information). The divergence of *B. arietans* likely occurred in the Zambezian and Southern regions, with the divergence and diversification of the *Calechidna* clade accompanied by a transition into the Southern biogeographic region. The maximum likelihood topology differed from the species tree at the deepest node (placement of *B. arietans* and *B. worthingtoni*), resulting in the geographic origin of *Bitis* estimated as the Southern region with subsequent northward transition to the Zambezian region, followed later by a return transition to the Southern region (Fig. S13, Table S5 in Supporting Information). None of the analyses suggested a North African nor a Eurasian origin.

DISCUSSION

In Africa, groups that have undergone habitat-dependent evolution should show phylogenetic signatures that match the expansion of open habitats starting in the late Oligocene and the particularly notable habitat shifts in the Miocene. Our results show that the genus *Bitis* diverged from sister clades in the early Oligocene, and this does not seem to be in response to the reduction of forest/woodland, given that most other African viper genera are also found in open habitat. Consistent with this, our analysis shows the ancestral state for *Bitis* as open habitat. Therefore, habitat-dependent evolution does not seem to be the initial driver of diversification within the African viperines, nor did it initiate the divergence of *Bitis* from other viperines. The majority of species level diversification within *Bitis* began in the late Miocene, with noteworthy divergence events occurring more recently for the species in hyper-arid regions. We found four well-supported clades that correspond to the currently recognised subgenera, and our phylogeny shows at least one cryptic taxon within *Bitis caudalis* and possibly *B. atropos*.

Is the evolution of *Bitis* habitat dependent?

We hypothesised that open habitat *Bitis* lineages (subgenera: *Calechidna*, *Keniabitis* and *Bitis*) diverged either in response to the initial but gradual aridification of Africa (Eocene/Oligocene), or later during the rapid mid-Miocene expansion of open habitats. The ancestral state for the genus is an open habitat at the basal node (median estimated age 26.2 mya, 95% credibility interval 20.6–33.7 mya), with one shift to forest by *Macrocerastes* in the mid-Miocene. Given that the ancestral state is open habitat, the origin of *Bitis* does not appear to be a case of habitat-dependent evolution in response to a shift from closed to open habitats, because the genus emerged at a time when open habitats already existed. Indeed, it is likely that closed or dense canopy forest and woodland formed a mosaic with open habitats (Linder, 2017) providing ample opportunity for diversification into open vegetation. The ‘forest-living’ ancestral condition for the entire subfamily is itself questionable, as most other viperine lineages except *Atheris* and some *Causus* inhabit primarily open formations. It is highly likely then, that Viperinae evolved in an open habitat setting in the Oligocene, with multiple shifts into forest by certain lineages (i.e. *Atheris* and subgenus *Macrocerastes*).

Although the origin of *Bitis* in the Oligocene is inconsistent with habitat-dependent evolution, within the genus there are indications of habitat-dependent diversification.

Vicariance initiated by the fragmentation of forest during the late-Miocene and Pliocene may have contributed to cladogenesis within the forest-dwelling *Macrocerastes*. Furthermore, the mid-Miocene divergence of the *Calechidna* clade coincides with the intensification of the Benguela oceanic current and associated development of the arid conditions in the west, including establishment of the Namib Desert (Scott & Anderson, 1997; Udeze & Oboh-Ikuenobe, 2005). All four arenicolous *Calechidna* lineages occur in the west, suggesting they shifted to the arid niche as it became available. Diversification within *Calechidna* is more recent, within the last ca. 5 mya. This corresponds well to the late Miocene/Pliocene shift from moist woodland and forest to the present day arid open habitat conditions in Namaqualand and the Karoo (Scott & Anderson, 1997; see Fig. S14 in Supporting Information for these localities). It is likely that an arid-living ancestral clade from the Namib region (*B. peringueyi* and *B. caudalis* L1) diversified and shifted to the more southern central Karoo (*B. caudalis* L2) and west coast Namaqualand (*B. schneideri*) as habitat became more xeric. However, throughout the Pleistocene the climate varied widely due to glacial cycling. Indeed, the central Karoo is considered to have high 'climate velocity', whereby the biome has shifted in position and extent during the Pleistocene (Tolley *et al*., 2014). The current biomes have apparently been relatively stable in extent through the Holocene (Scott & Anderson, 1997). Although the region has been climatically dynamic, there has been a long-term aridification trend which has undoubtedly influenced cladogenesis within the *Calechidna*. The formation of the arid west and Namib Desert has also been linked to evolutionary diversification in lizards (Lamb & Bauer, 2003, 2006; Makokha *et al*., 2007; Edwards *et al*., 2016), and this extreme environment certainly must have played a role in speciation and adaptation of arid-living fauna.

In addition to habitat factors, divergence timings within *Bitis* also correspond with geological events. Specifically, the divergence of *B. parviocula* from its sister clade coincides with the extension of the Main Ethiopian rift which began around 11 mya (postdating the initial rifting of the Red Sea/Gulf of Aden in the late Oligocene; Wolfenden *et al*., 2004). Considering the limited distribution of *B. parviocula* along the Ethiopian Rift, this result strongly suggests a causal role for these geological processes in the origin of this species, as has been suggested for other East African squamate lineages (Matthee *et al*., 2004; Wüster *et al*., 2007; Tolley *et al*., 2011). It should be noted that genetic data for the newly described *B. harenna* is still lacking, but is essential to test this hypothesis. In contrast, however, *B. worthingtoni* currently has a limited distribution along the Kenyan Rift Valley but divergence from its sister clade considerably pre-dates the onset of rift formation and volcanism in Kenya, 16–20 mya (Chorowicz, 2005), suggesting that these geological events were not involved in the divergence of this taxon.

We acknowledge that our dating analysis was calibrated using a single Eurasian viper fossil, so our interpretations regarding timing of events should be treated with some caution. However, other molecular phylogenies that include vipers also place the divergence of *Bitis* from other vipers within the Oligocene (ranging between 35–40 mya; Wüster *et al*., 2008, Alencar *et al*., 2016), corresponding with our own analysis that suggests a divergence around 31.9 mya (95% CI 26–40 mya). Inclusion of additional calibration points may refine the diversification dates within *Bitis*, but it is unlikely that the dating would shift so substantially as to alter our main interpretations.

The geographic origin of *Bitis* unfortunately remains elusive, in part due to the differing topologies for the species tree and the maximum likelihood tree at the deepest node. The species tree analysis showed a Zambezian+Ethiopian/Somalian ancestral area, whereas the ML topology suggests a southern African origin. The analysis would likely be improved with the addition of missing genera (*Atheris*, *Eristicophis*, *Macrovipera*, *Montatheris*, *Proatheris*, *Pseudocerastes*) and species (*B. heraldica, B. harenna*). The Zambezian and North African regions experienced substantial reduction in forest (opening of habitat) during the Oligocene (Morley, 2007). Both analyses are in agreement that the genus did not originate in North Africa, but rather in the south/eastern region of the continent, with the Zambezian region playing an important role. Therefore, we suggest that the opening of habitat in the Zambezian region initiated the diversification of this genus. It also appears that the common ancestor for the crown groups occurred in the Zambezian region (ca. 20–25 mya), and then split into a southern African clade (*Calechidna*) and a more widespread clade centred in the eastern-central portion of the continent (*Macrocerastes*).

Phylogeny and systematics of *Bitis*

Our results provide new information on the phylogeny and systematics of *Bitis.* A key question which has remained equivocal despite numerous phylogenetic studies is relationships among the *Bitis* subgenera, specifically the relative positions of *Keniabitis* and the *B. arietans* lineage (Lenk *et al.,* 1999; Wüster *et al*., 2008, Alencar *et al.,* 2016). Through multispecies coalescent analysis of mitochondrial and three nuclear loci we were able to resolve this relationship with high posterior support, placing *B. arietans* as sister to *Macrocerastes* and *Calechidna*, with *Keniabitis* in turn sister to this clade. Achieving this robust phylogenetic hypothesis for *Bitis* subgenera will benefit future studies on the evolution and diversification of this group.

Furthermore, we suggest that current taxonomy may not fully capture species diversity within the subgenus *Calechidna*. The four samples of *B. caudalis* analysed comprise two divergent and polyphyletic mitochondrial lineages. Multispecies coalescent analysis of these lineages suggests that *B. caudalis* L2 and *B. schneideri* (both from southwestern South Africa) share a recent common ancestry, whereas *B. caudalis* L1 and *B. peringueyi* (both from western Namibia) (Fig. S15 in Supporting Information) share a recent common ancestry. The maximum likelihood analysis however, differed for these relationships although each of these clades was still supported as distinct. *Bitis caudalis* is widespread across south-western Africa, occurring from southern Angola southwards to the Western Cape Province of South Africa, and eastwards to southern Zimbabwe. Because our sampling was limited, we cannot make firm conclusions regarding these relationships. Indeed, a comprehensive phylogeographic analysis of this widespread taxon is a priority for future studies on *Bitis*, particularly as the two analyses showed slightly different relationships between the clades.

Further indication of potentially cryptic species diversity was found among *B. atropos* populations. Specifically, the Zimbabwean *B. atropos* possessed unique alleles for two nuclear markers (Fig. 2b), and exhibited significant levels of mitochondrial divergence from conspecific samples (all from the Western Cape, South Africa), comparable with divergences of other interspecific rather than intraspecific relationships within *Calechidna* (Fig. 2a). *Bitis*

atropos has a fragmented distribution with populations occurring along the Cape Fold Mountains in the Western and Eastern Cape Provinces of South Africa, and additional allopatric populations in the KwaZulu-Natal and Mpumalanga provinces of South Africa, and in Zimbabwe. It was hypothesised that these isolated populations represent a assemblage of sibling species (Branch, 1999). It was later shown that the *B. atropos* 'complex' comprises a suite of cryptic species that apparently originated in stepwise fashion from north to south, associated with isolation of montane grassland habitats of the Great Escarpment (Kelly *et al.,* 2011). Together with our results, this highlights *B. atropos* as an important focus for future research efforts.

The *cornuta-inornata* complex comprises five morphologically and ecologically differentiated species (Branch, 1999), which our molecular dating analysis shows to have radiated much more recently than other *Bitis* clades (within the last ca. 1.2 my). Analysis of mitochondrial sequences and the maximum likelihood analysis recovered *B. albanica* and *B. rubida* as polyphyletic, and these together showed little differentiation from *B. inornata*. Sharing of nuclear alleles was also evident among these three taxa as well as among the other species in the complex, *B. armata* and *B. cornuta*. These genetic patterns are consistent with a recent radiation of these species, and any taxonomic interpretations based on our limited sampling would be premature. The relationships between these taxa might become better understood with denser sampling of individuals and additional genetic loci.

Above the species-level, previous discussions of *Bitis* systematics have considered their higher level taxonomy, specifically whether the four subgeneric clades may warrant elevation to genus level (Herrmann & Joger, 1997; Lenk *et al*., 1999). Changes in nomenclature are justified in cases where current taxonomy does not adequately portray evolutionary relationships, but this must be balanced against the potential negative impacts of taxonomic changes on the wider scientific community. Given the strong support for monophyly of the genus *Bitis* as currently defined, we share the view of Wüster *et al.* (2008) that splitting of this historically stable group would only serve to confuse the nomenclature and hinder information retrieval without significantly enhancing our understanding of the evolutionary history of the genus. The continued recognition of the *Bitis* subgenera, however, does provide an effective way of highlighting the major evolutionary and ecological divisions within the genus whilst avoiding any potentially negative effects of generic reassignment. Overall, this results in a more information-rich classification (Wallach *et al*., 2009).

CONCLUSION

Our analysis was limited to a dichotomy of open/closed habitats, yet the vegetation of Africa was surely more complex through space and time. Therefore, we are limited to interpretations relating only to broad scale patterns; yet diversification within *Bitis*, and indeed within viperines, could easily have been driven by nuances rather than the generalities that are characterise our study. Until such time that the complexities of African paleo-vegetation are revealed, broad patterns over large time scales will characterise our best knowledge. Overall, we show that the diversification of *Bitis* likely began in open habitats in the late Oligocene/early Miocene, prior to the major expansion of such habitats in the mid-Miocene. This contrasts strongly with open habitat mammalian lineages which are shown by the fossil record to have diversified much later, following the expansion of C_4

grasslands in the late Pliocene and Pleistocene (Vrba, 1992; Wesselman, 1985; Bobe *et al*., 2002; Bobe & Behrensmeyer, 2004). Overall, our results highlight the need for taxonomic breadth in achieving a holistic understanding of faunal evolution in Africa, as well as for fine-scale analyses that aim to incorporate subtleties of vegetation and climatic dynamics.

Table 1. Taxonomy of *Bitis* and habitat preference for each species.

Subgenus	**species**		**habitat**
Macrocerastes	*B. gabonica*	East African Gaboon Adder	Tropical and montane forest
	B. rhinoceros	West African Gaboon Adder	
	B. nasicornis	Rhinoceros Viper	
	B. parviocula	Ethiopian Mountain Adder	
	*B. harenna**	Bale Mountains Adder	
Calechidna	*B. albanica*	Albany Adder	lowland and montane rocky or gravely grassland, karroid and Sclerophyllous scrub
	B. armata	Southern Adder	
	B. atropos	Berg Adder	
	B. cornuta	Many-horned Adder	
	*B. heraldica**	Angolan Adder	
	B. inornata	Plain Mountain Adder	
	B. rubida	Red Adder	
	B. xeropaga	Desert Mountain Adder	
	B. caudalis Lineage 1	Horned Adder	sandy savanna & karroid scrub and alluvial soils
	B. caudalis Lineage 2		
	B. peringueyi	Peringuey's Adder	Namib sand sea
	B. schneideri	Namaqua Dwarf Adder	coastal sand dunes
Bitis (type subgenus)	*B. arietans* complex	Puff Adder	open savanna, grassland and karroid scrub absent from forest and desert
Keniabitis	*B. worthingtoni*	Kenya Horned Viper	montane grassland and scrub

*Subgeneric assignment not confirmed by genetic analysis

Figure 1 a) *Bitis* MSC species tree. Nodes are centred on the median age from the posterior sample, and the 95% CIs indicated by the blue bars. Node support values are Bayesian posterior clade probabilities. Support values are from the three locus analysis (those preceded by asterisks were supported in the four locus analysis). The major subgeneric *Bitis* clades are indicated to the right of the figure and are coloured according to habitat preference. The general shift from forest (green) to open (yellow) habitats in the mid-Miocene is indicated, with inset maps showing rough extent of forest/woodland mosaic (stippled green) in the Oligocene and at present (blue indicates areas inundated by sea). The ancestral character states at major nodes are shown by coloured circles. b) Maximum likelihood bootstrap consensus tree for the concatenated four gene analysis, with terminal tips collapsed for each clade/species. Bootstrap values are given for nodes with > 70% support. The topology differs from the species tree at the nodes indicated by arrows. For both figures, outgroup taxa have been removed for clarity but are shown in Supporting information.

Figure 2 a) Mitochondrial gene tree estimated in the three-locus MSC analysis for *Bitis*. Filled circles at nodes indicate Bayesian clade support of 1.0, whereas values < 1.0 are given numerically. b) Matrix of *Bitis* species showing instances of shared alleles (filled squares) for the nuclear PRLR (below the diagonal) and UBN1 (above the diagonal) genes. Asterisks indicate species for which monophyly was supported by posterior probabilities $\geq$ 0.9 in the nuclear gene trees estimated in the three-locus MSC analysis for PRLR (vertical list, see Fig. S4 in Supporting Information) and UBN1 (horizontal list, refer to Fig. S5 in Supporting Information).

Figure 3 Ancestral area reconstruction for *Bitis*. Proportional likelihood values are shown for each node by coloured doughnut charts (colour codes match key). Area coding for each taxon/tip is indicated: A-Eurasia, B-North Africa, C-Congolian, D-Ethiopian/Somalian, E-Sudanian, F-Zambezian, G-Southern, and corresponds to the map of biogeographic regions for Africa (inset).

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Biosketches

Axel Barlow is interested in studying the evolutionary history of populations using DNA sequence data. His work encompasses a range of vertebrate taxa across a variety of geographic regions and temporal scales. He is also interested in the development of new laboratory and analytical approaches that can be applied to evolutionary questions.

Wolfgang Wüster is a herpetologist interested in the systematics, phylogeography and phylogeny of venomous snakes and the evolution of snake venoms.

Krystal A. Tolley is interested in understanding the historical processes that generate patterns of diversity in African reptiles using biogeographic and phylogenetic approaches.

Author Contributions A.B. and W.W. funded and designed the project. A.B. and C.M.R.K. carried out laboratory work. A.B., W.W. and K.A.T. analysed the data, interpreted the results and wrote the manuscript. All authors contributed to sampling and to the manuscript text.

Supporting Figures[1]

Figure S1. Paleo-vegetation in African during the Oligocene based on Morley 2007
Figure S2. Biogeographic regions of Africa based on Linder et al. 2012
Figure S3. Maximum likelihood tree for phased PRLR dataset
Figure S4. Maximum likelihood tree for unphased PRLR dataset
Figure S5. Maximum likelihood tree for phased UBN1 dataset
Figure S6. Maximum likelihood tree for unphased UBN1 dataset
Figure S7. MSC species tree from the three locus analysis (two linked mitochondrial, two unlinked nuclear)
Figure S8. Maximum likelihood tree for three locus analysis (two linked mitochondrial, two unlinked unphased nuclear)
Figure S9. MSC PRLR gene tree from the three locus analysis
Figure S10. MSC UBN1 gene tree from the three locus analysis
Figure S11. Subgenus level MSC species tree from the four locus analysis
Figure S12. Character state coding and results of ancestral characters state optimisation for habitat type

Figure S13. Character state coding and results of ancestral characters state optimisation for area using the maximum likelihood phylogeny
Figure S14. Map of southern Africa with place names indicated
Figure S15. Interpreted distributions of *Bitis caudalis*, *B. peringueyi* and *B. schneideri*

[1]Figures S3-S11 are available as nexus files on Dryad, which can be visualised interactively using FigTree or similar software.

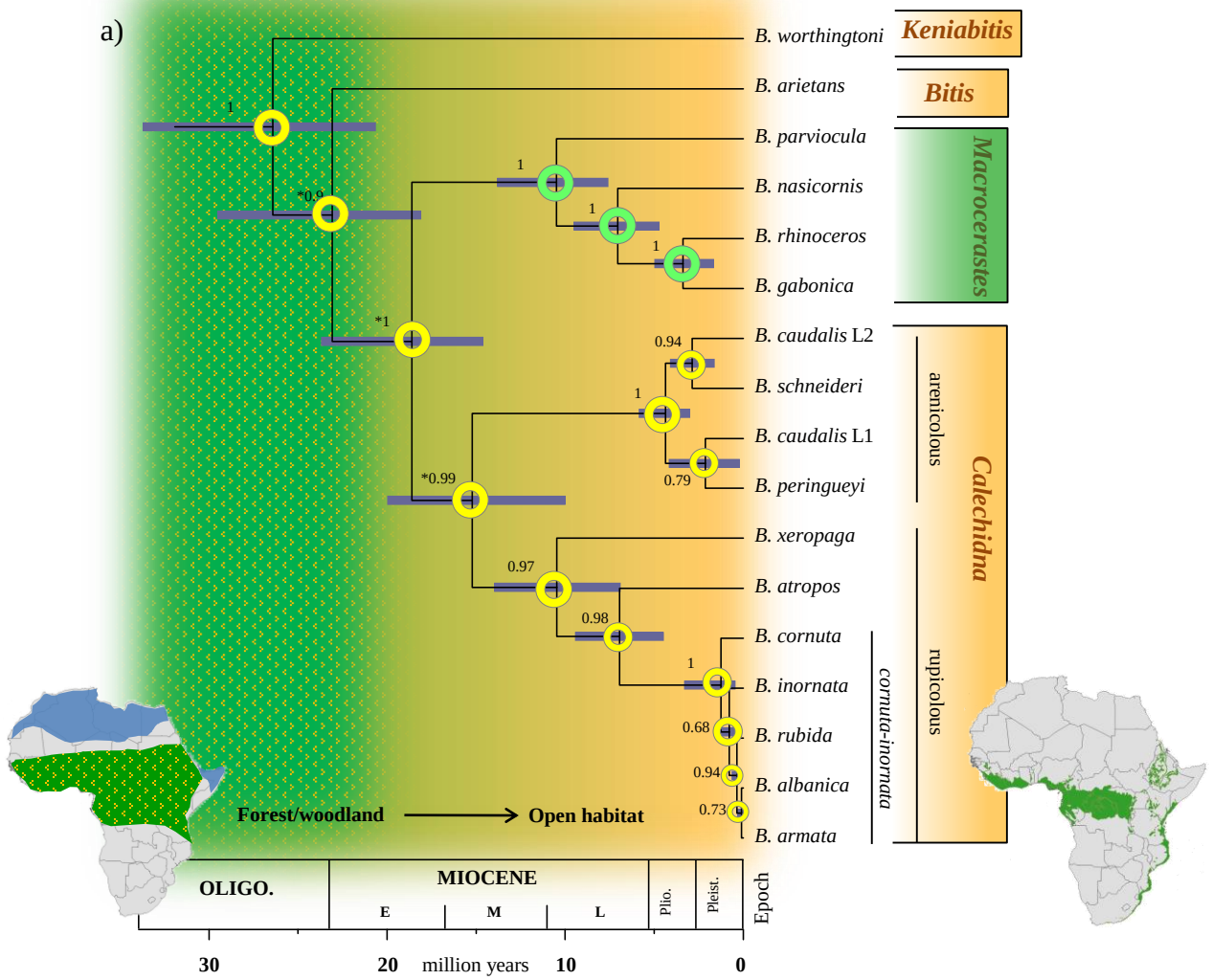
Supporting Tables
Table S1. Details of individuals sequenced for this study with corresponding GenBank accession numbers
Table S2. Ancestral area coding
Table S3. Ancestral range constraints
Table S4. Dispersal probabilities between regions at four time periods
Table S5. Proportional likelihoods for ancestral area reconstructions using a) the species tree, b) the maximum likelihood tree

Supporting data deposited in Dryad

mtDNA alignment as a nexus file in Dryad

PRLR alignment as a nexus file in Dryad

UBN1 alignment as a nexus file in Dryad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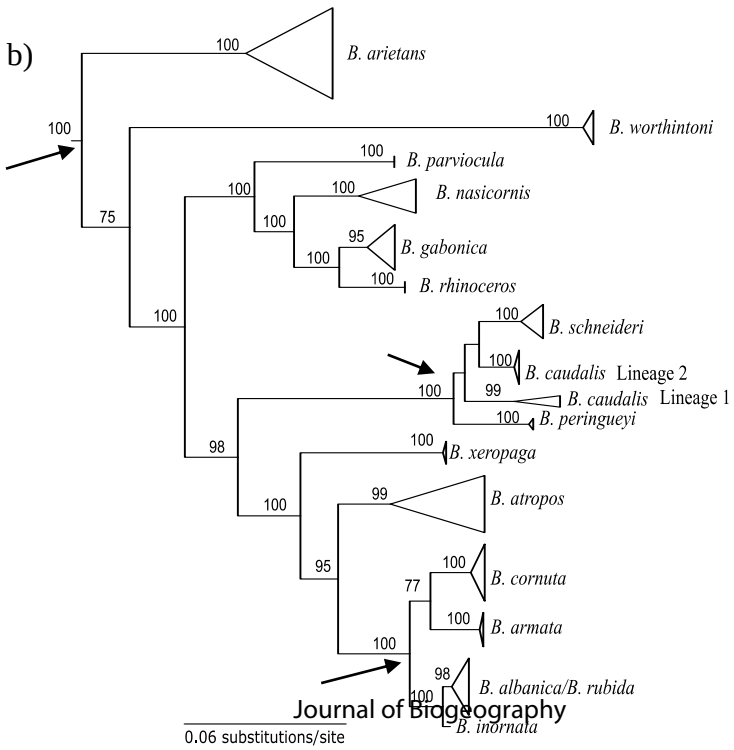
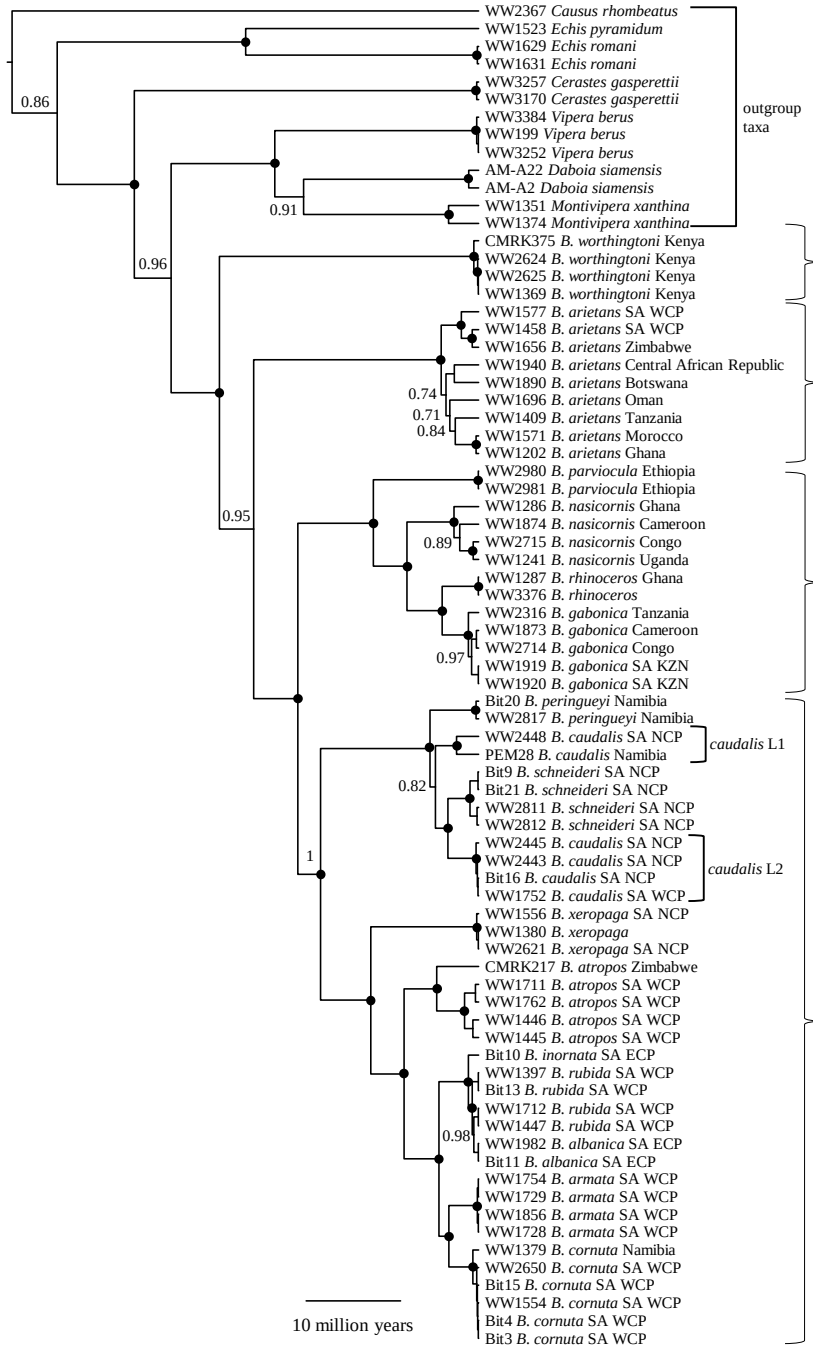
B. arietans
B. parviocula
B. nasicornis
B. rhinoceros
B. gabonica
B. caudalis L2
B. schneideri
B. caudalis L1
B. peringueyi
B. xeropaga
B. atropos
B. cornuta
B. inornata
B. rubida
B. albanica
B. armata
Keniabitis
Bitis
Macroscelides
Calechidna
arenicolous
rupicolous
cornuta-inornata
1
*0.9
*1
0.94
0.79
*0.99
0.97
0.98
0.68
0.94
0.73
Forest/woodland
Open habitat
OLIGO.
MIOCENE
E
M
L
Plio.
Pleist.
Epoch
30
20
million years
10
0


b)
B. arietans
B. worthintoni
B. parviocula
B. nasicornis
B. gabonica
B. rhinoceros
B. schneideri
B. caudalis Lineage 2
B. caudalis Lineage 1
B. peringueyi
B. xeropaga
B. atropos
B. cornuta
B. armata
B. albanica/B. rubida
B. inornata
0.06 substitutions/site

a)


WW2367 Causus rhombeatus
WW1523 Echis pyramidum
WW1629 Echis romani
WW1631 Echis romani
WW3257 Cerastes gasperettii
WW3170 Cerastes gasperettii
WW3384 Vipera berus
WW199 Vipera berus
WW3252 Vipera berus
AM-A22 Daboia siamensis
AM-A2 Daboia siamensis
WW1351 Montivipera xanthina
WW1374 Montivipera xanthina
outgroup taxa
0.86
0.91
0.96
CMRK375 B. worthingtoni Kenya
WW2624 B. worthingtoni Kenya
WW2625 B. worthingtoni Kenya
WW1369 B. worthingtoni Kenya
Keniabitis
WW1577 B. arietans SA WCP
WW1458 B. arietans SA WCP
WW1656 B. arietans Zimbabwe
WW1940 B. arietans Central African Republic
WW1890 B. arietans Botswana
WW1696 B. arietans Oman
WW1409 B. arietans Tanzania
WW1571 B. arietans Morocco
WW1202 B. arietans Ghana
0.74
0.71
0.84
Bitis
WW2980 B. parviocula Ethiopia
WW2981 B. parviocula Ethiopia
WW1286 B. nasicornis Ghana
WW1874 B. nasicornis Cameroon
WW2715 B. nasicornis Congo
WW1241 B. nasicornis Uganda
WW1287 B. rhinoceros Ghana
WW3376 B. rhinoceros
WW2316 B. gabonica Tanzania
WW1873 B. gabonica Cameroon
WW2714 B. gabonica Congo
WW1919 B. gabonica SA KZN
WW1920 B. gabonica SA KZN
0.95
0.89
0.97
Macrocerastes
Bit20 B. peringueyi Namibia
WW2817 B. peringueyi Namibia
WW2448 B. caudalis SA NCP
PEM28 B. caudalis Namibia
caudalis L1
Bit9 B. schneideri SA NCP
Bit21 B. schneideri SA NCP
WW2811 B. schneideri SA NCP
WW2812 B. schneideri SA NCP
0.82
WW2445 B. caudalis SA NCP
WW2443 B. caudalis SA NCP
Bit16 B. caudalis SA NCP
WW1752 B. caudalis SA WCP
caudalis L2
1
WW1556 B. xeropaga SA NCP
WW1380 B. xeropaga
WW2621 B. xeropaga SA NCP
CMRK217 B. atropos Zimbabwe
WW1711 B. atropos SA WCP
WW1762 B. atropos SA WCP
WW1446 B. atropos SA WCP
WW1445 B. atropos SA WCP
Calechidna
Bit10 B. inornata SA ECP
WW1397 B. rubida SA WCP
Bit13 B. rubida SA WCP
WW1712 B. rubida SA WCP
WW1447 B. rubida SA WCP
WW1982 B. albanica SA ECP
Bit11 B. albanica SA ECP
0.98
WW1754 B. armata SA WCP
WW1729 B. armata SA WCP
WW1856 B. armata SA WCP
WW1728 B. armata SA WCP
WW1379 B. cornuta Namibia
WW2650 B. cornuta SA WCP
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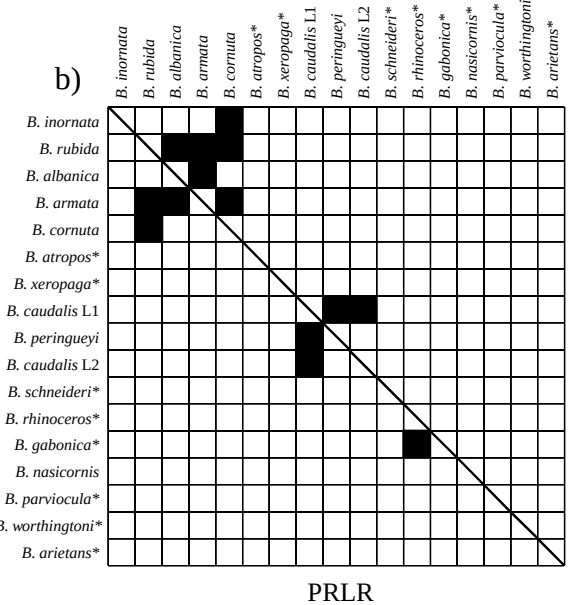
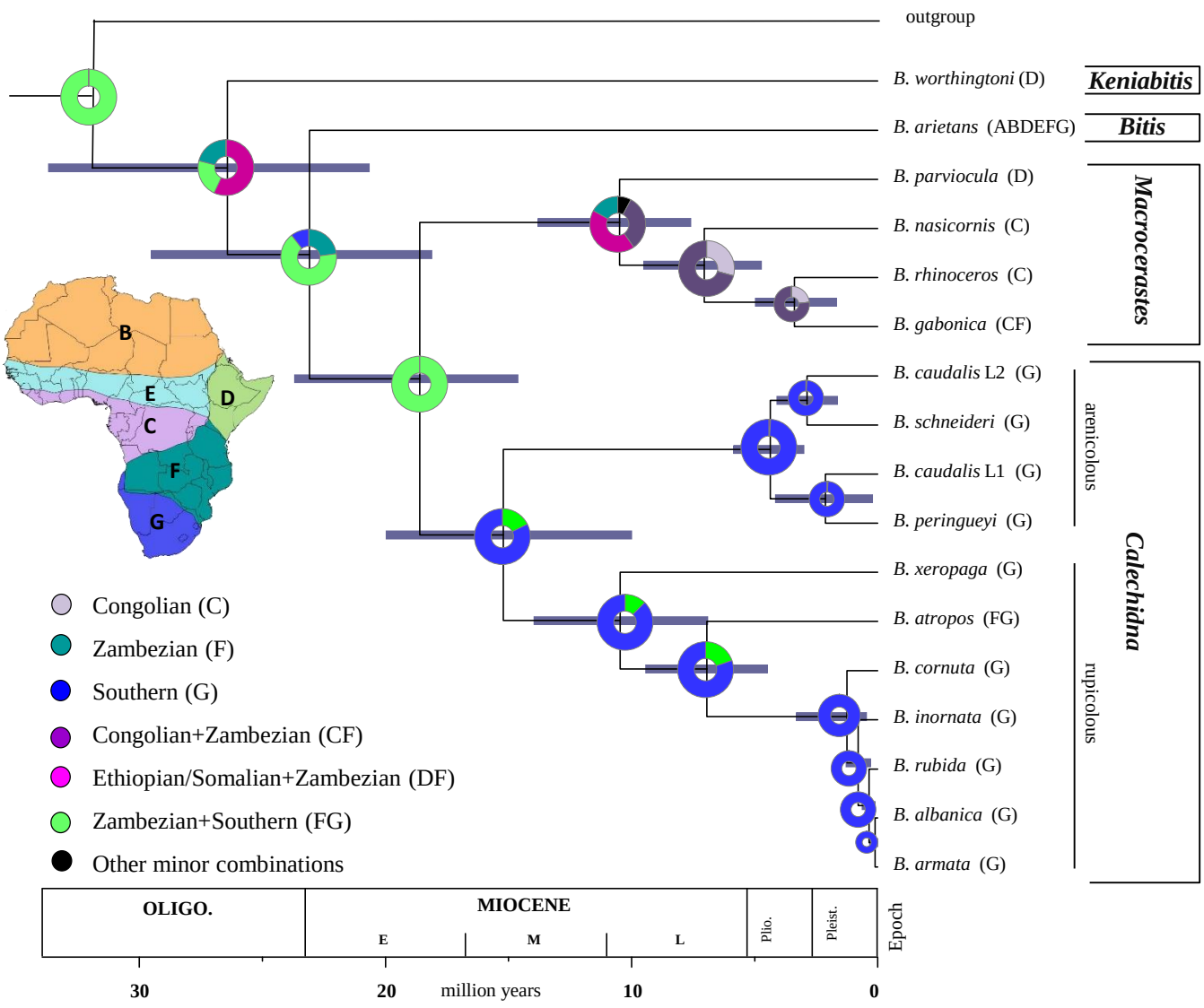
Bit3 B. cornuta SA WCP
10 million years

b)


B. inornata
B. rubida
B. albanica
B. armata
B. cornuta
B. atropos*
B. xeropaga*
B. caudalis L1
B. peringueyi
B. caudalis L2
B. schneideri*
B. rhinoceros*
B. gabonica*
B. nasicornis*
B. parviocula*
B. worthingtoni*
B. arietans*
UBN1
PRLR


outgroup
B. worthingtoni (D)
B. arietans (ABDEFG)
B. parviocula (D)
B. nasicornis (C)
B. rhinoceros (C)
B. gabonica (CF)
B. caudalis L2 (G)
B. schneideri (G)
B. caudalis L1 (G)
B. peringueyi (G)
B. xeropaga (G)
B. atropos (FG)
B. cornuta (G)
B. inornata (G)
B. rubida (G)
B. albanica (G)
B. armata (G)
Keniabitis
Bitis
Macrocerastes
Calechidna
arenicolous
rupicolous
B
E
D
C
F
G
Congolian (C)
Zambezian (F)
Southern (G)
Congolian+Zambezian (CF)
Ethiopian/Somalian+Zambezian (DF)
Zambezian+Southern (FG)
Other minor combinations
OLIGO.
MIOCENE
E
M
L
Plio.
Pleist.
Epoch
30
20
million years
10
0

Supporting Information
Journal of Biogeography

Ancient habitat shifts and organismal diversification are decoupled in the African viper genus Bitis (Serpentes: Viperidae).

Axel Barlow, Wolfgang Wüster, Christopher M. R. Kelly, William R. Branch, Tony Phelps and Krystal A. Tolley

Supporting Figures[1]

[1]Figures S3-S11 are available as nexus files on Dryad, which can be visualised interactively using FigTree or similar software.

Supporting Tables

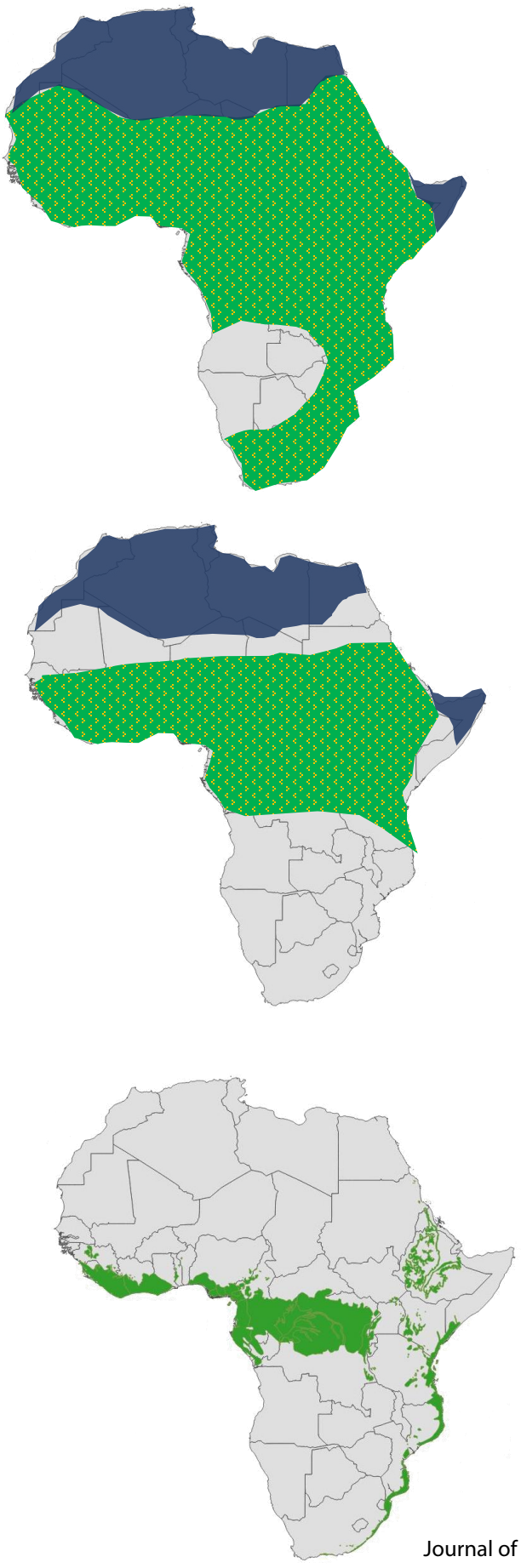


Figure S1. Approximate position and extent of paleo-vegetation in Africa during the Early Eeocene, 54-48 mya (top), the Oligocene 34-23 mya (middle) based on Morley 2007. Bottom map shows present day forest extent. Stippled green indicates the *rough* extent of forest/woodland/mosaic ("closed canopy"), blue indicates approximate areas inundated by sea, and grey indicates areas under more open vegetation.

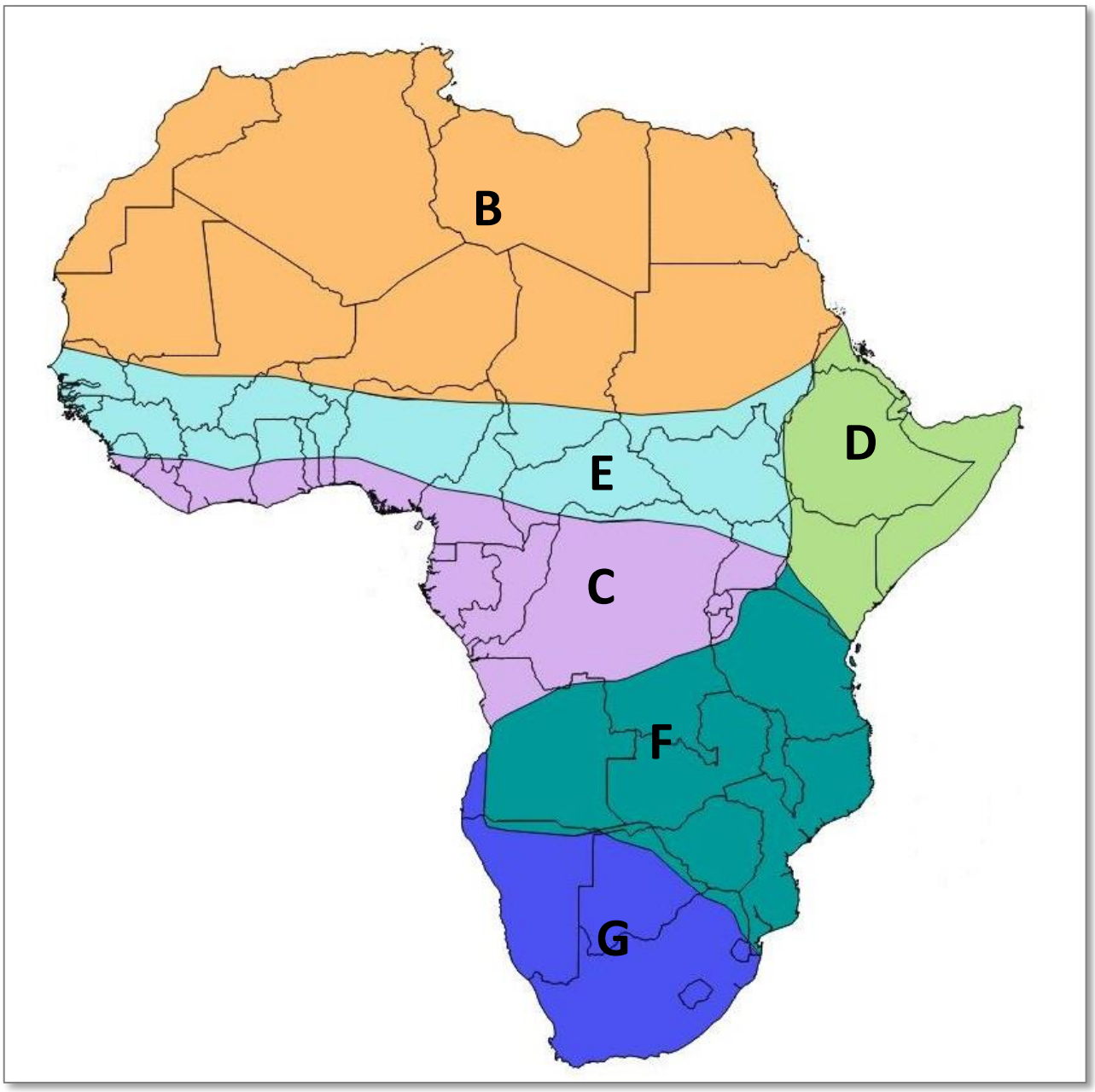


Figure S2. Biogeographic regions of Africa. These areas were used for coding terminal tips in the ancestral area reconstruction. B-North Africa, C-Congolian, D-Ethiopian/Somalian, E-Sudanian, F-Zambezian, G-Southern. Regions are re-drawn after Linder *et al*. 2012.

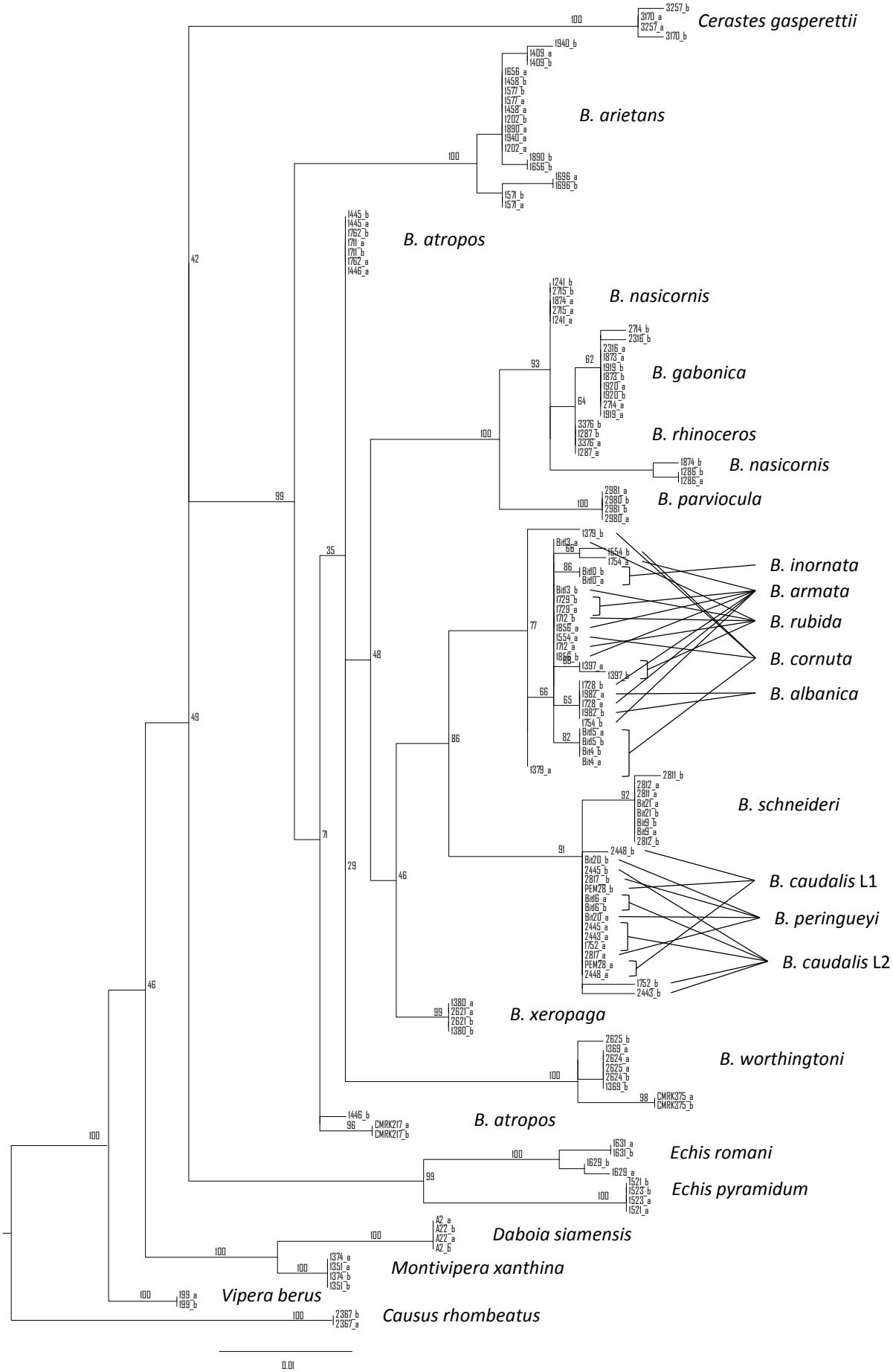


Figure S3. Maximum likelihood tree for phased PRLR dataset. Phased individuals indicated as 'a' and 'b' for each allele.

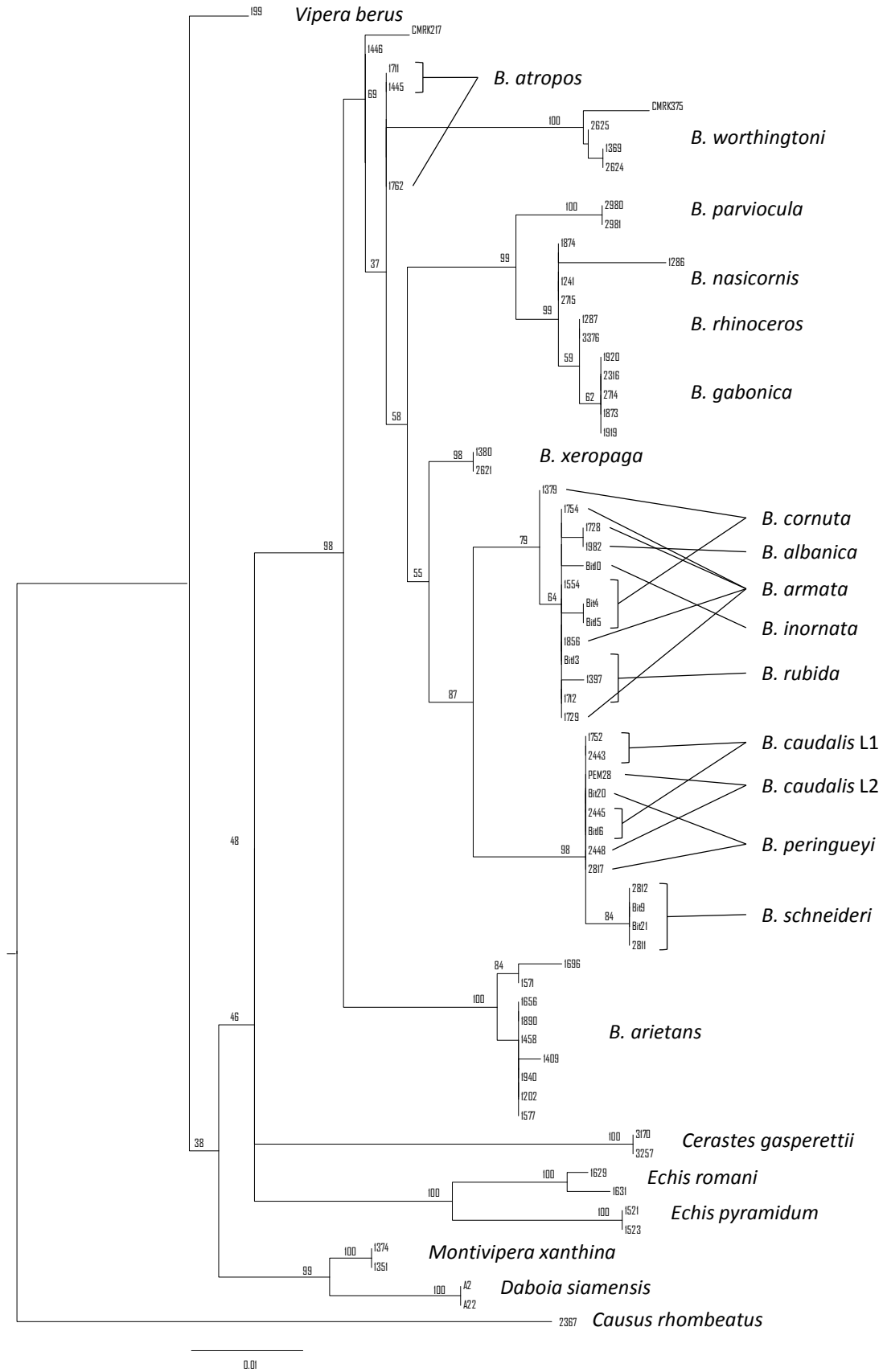


Figure S4. Maximum likelihood tree for unphased PRLR dataset.

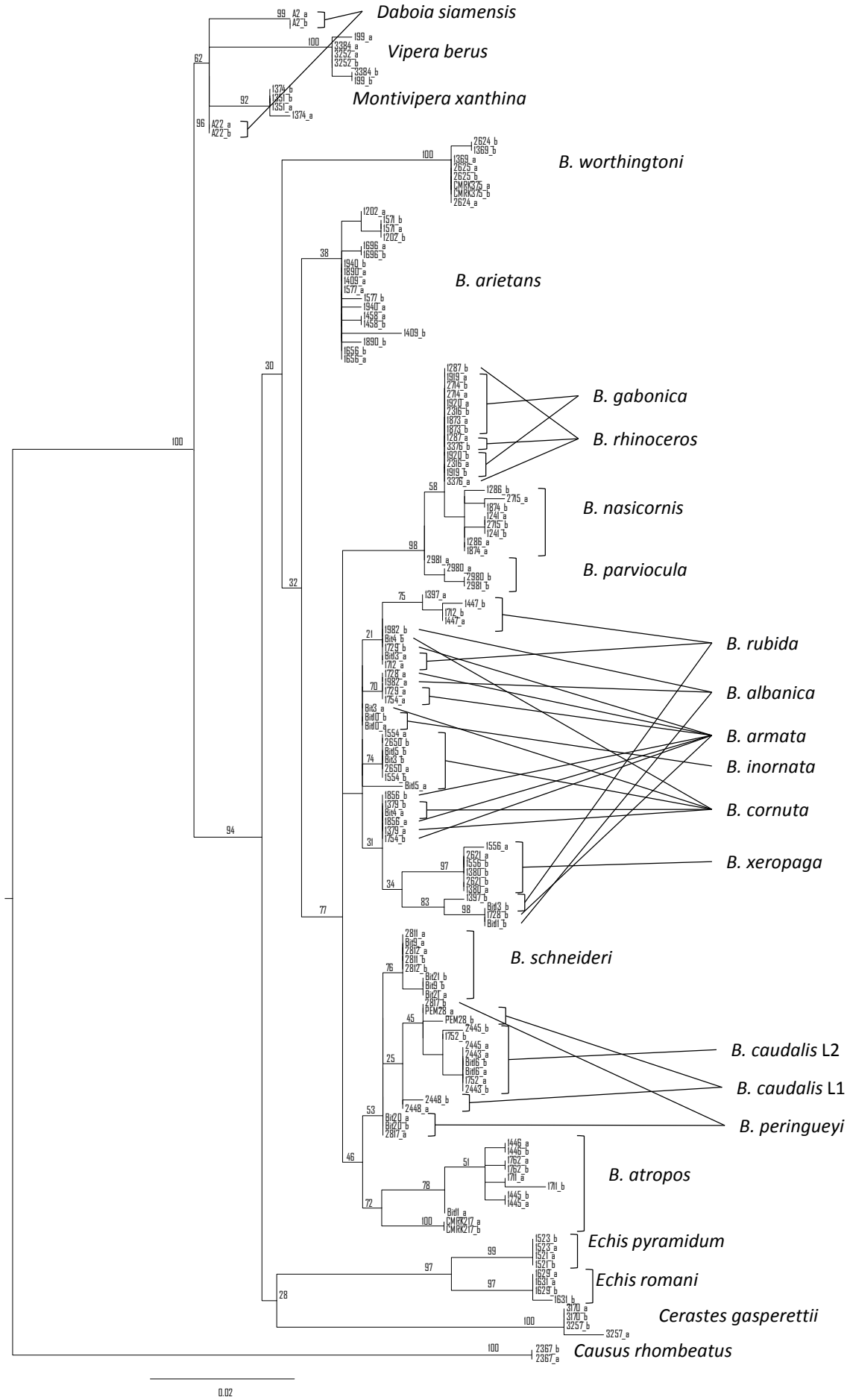


Figure S5. Maximum likelihood tree for phased UBN1 dataset. Phased individuals indicated as ‘a’ and ‘b’ for each allele.

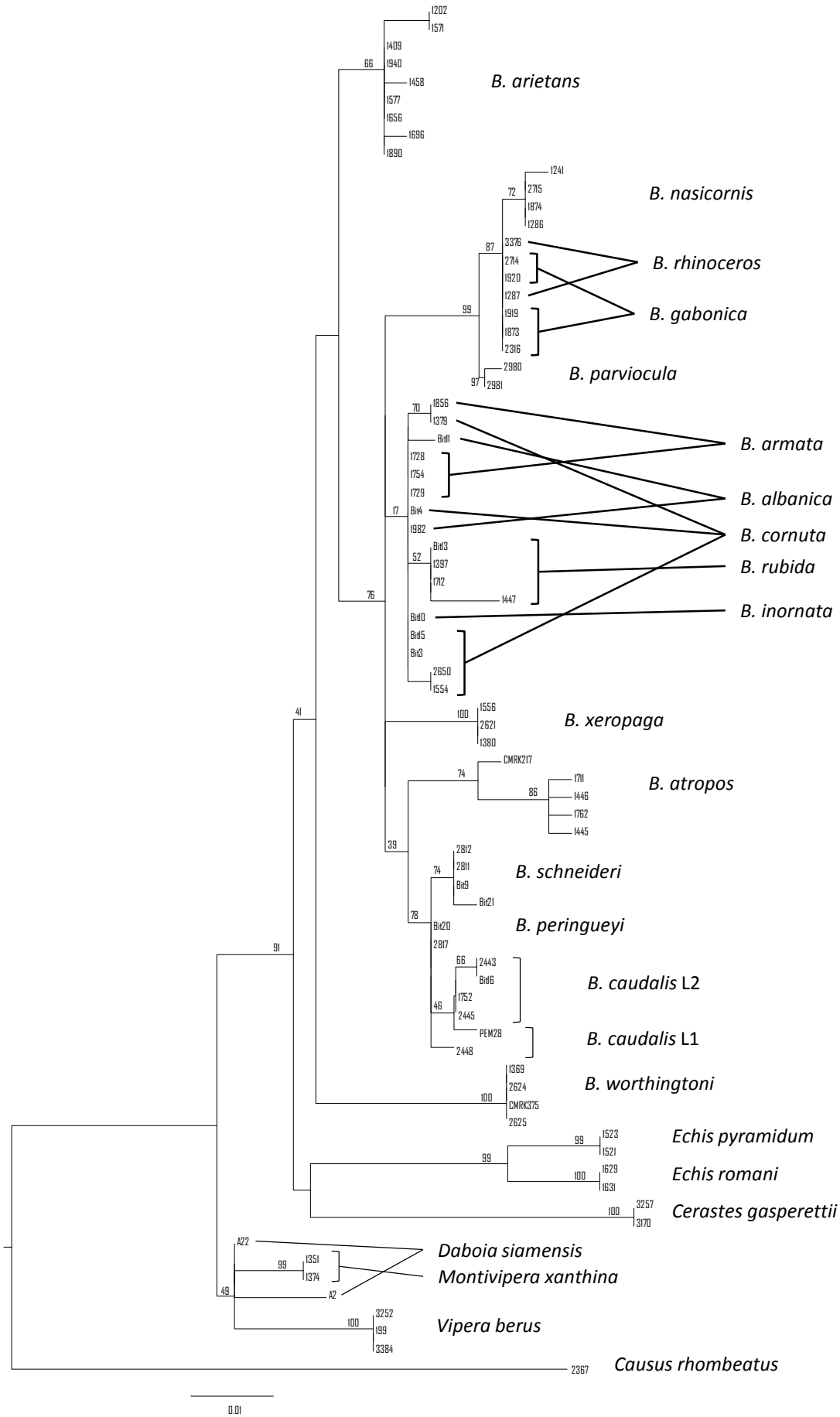


Figure S6. Maximum likelihood tree for unphased UBN1 dataset. Sample numbers, species names and sample sites have been abbreviated.

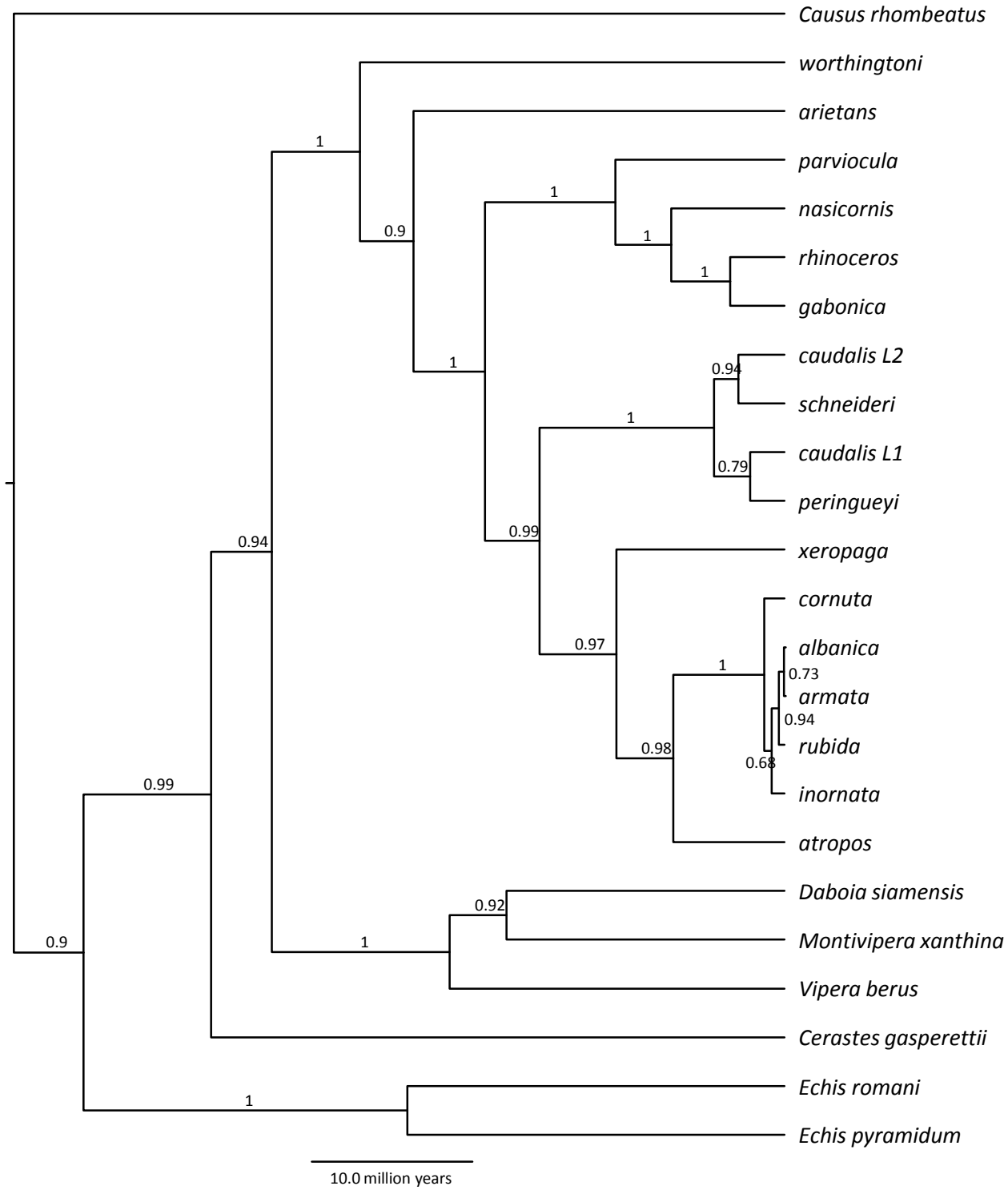


Figure S7. MSC species tree from the three locus analysis (two linked mitochondrial, two unlinked nuclear). Posterior probabilities for each node are given.

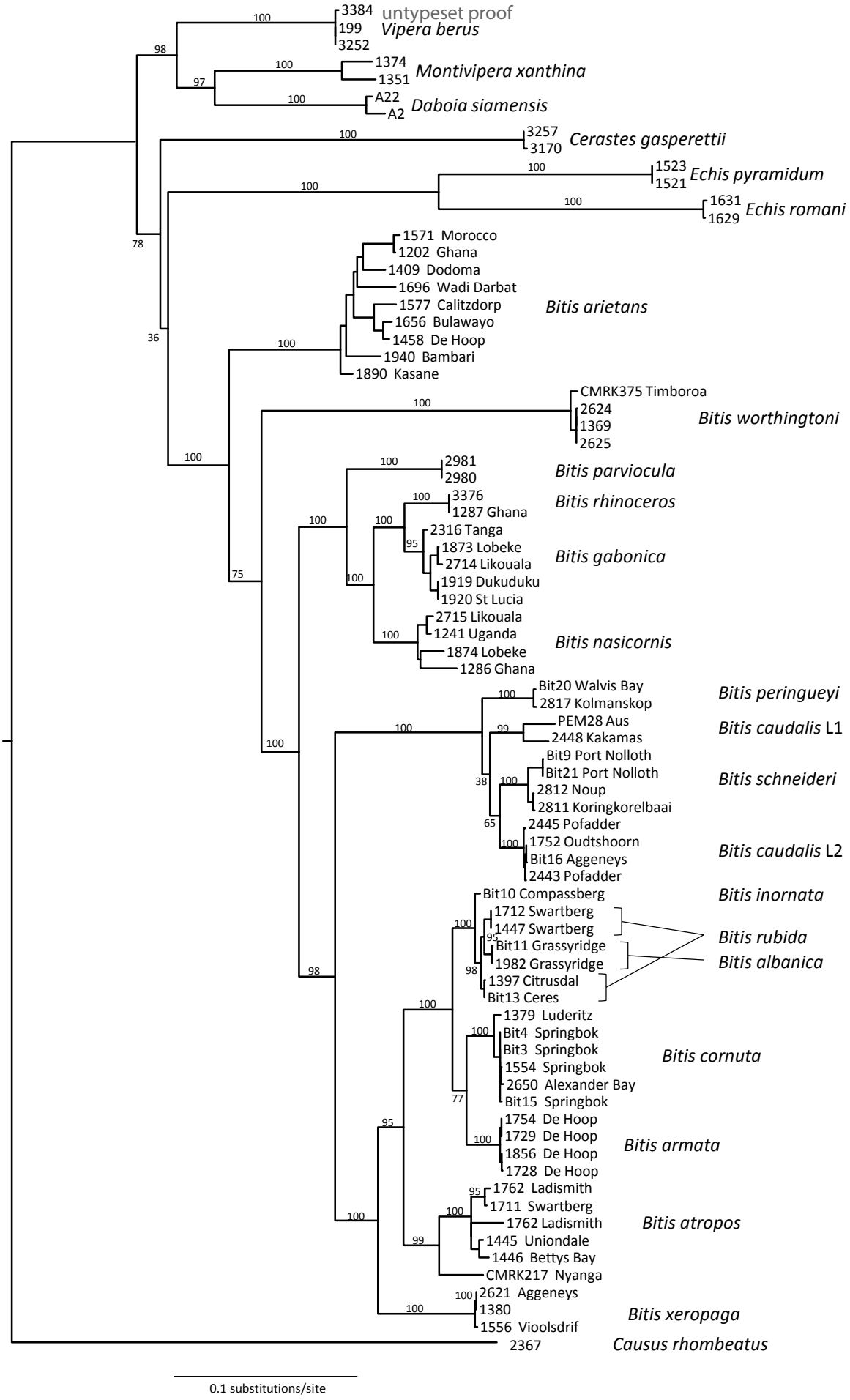


Figure S8. Maximum likelihood tree for four gene concatenated (two linked mitochondrial, two unlinked nuclear). Bootstrap values for each node are given.

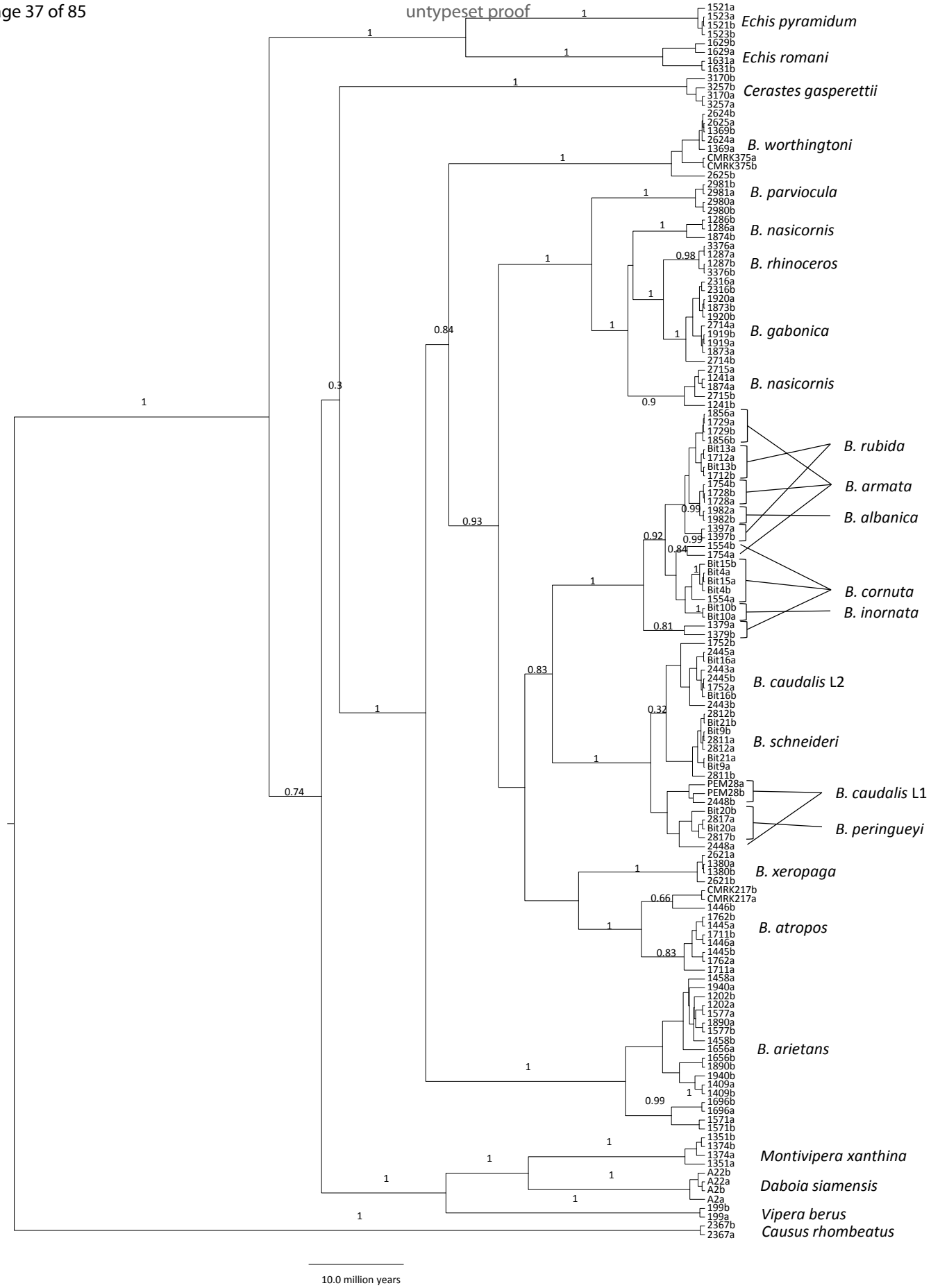


Figure S9. Phylogeny of the PRLR gene estimated in the three-locus MSC analysis (phased dataset). Posterior probabilities are given at each node.

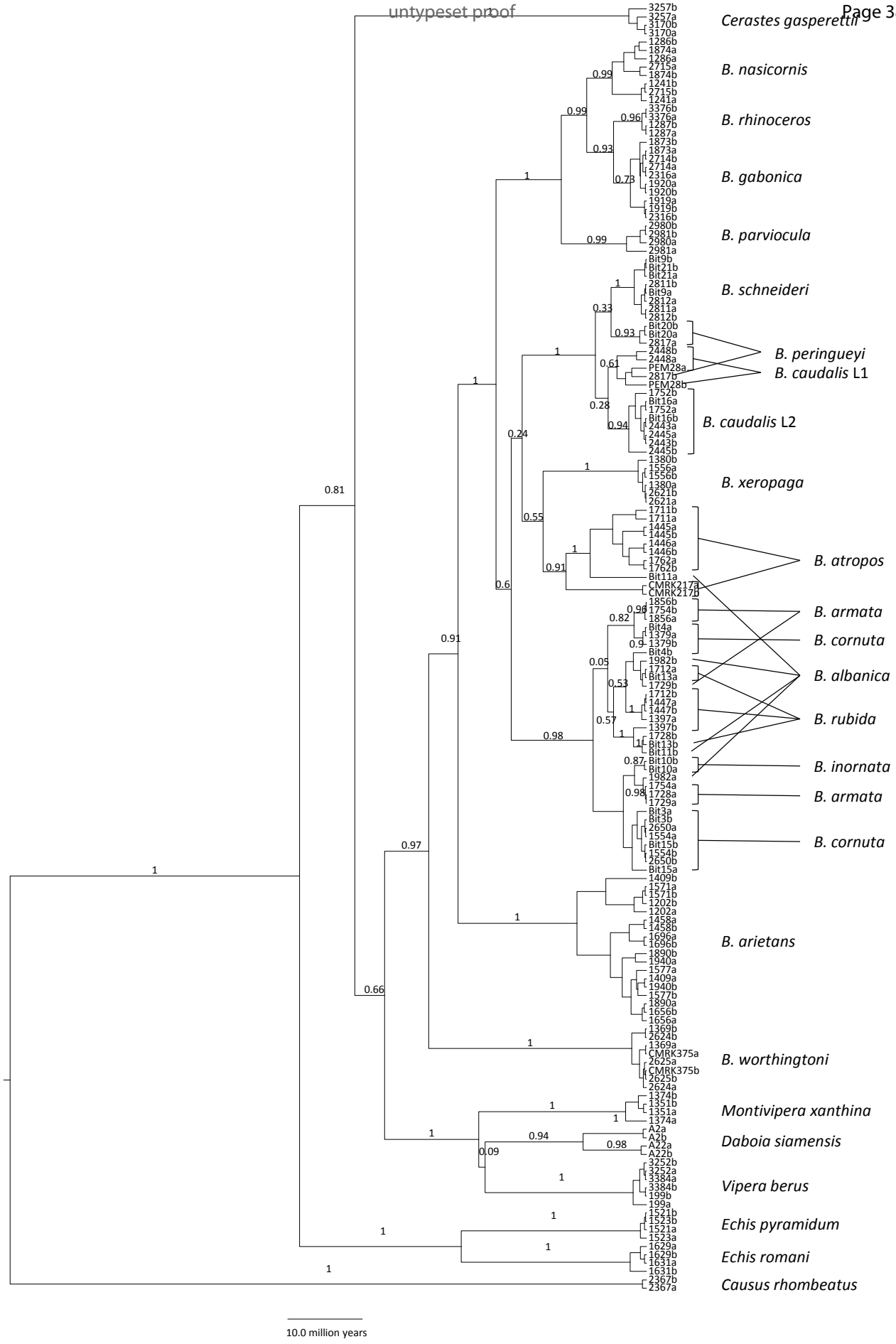


Figure S10. Phylogeny of the UBN1 gene estimated in the three locus MSC analysis (phased dataset). Posterior probabilities are given at each node.

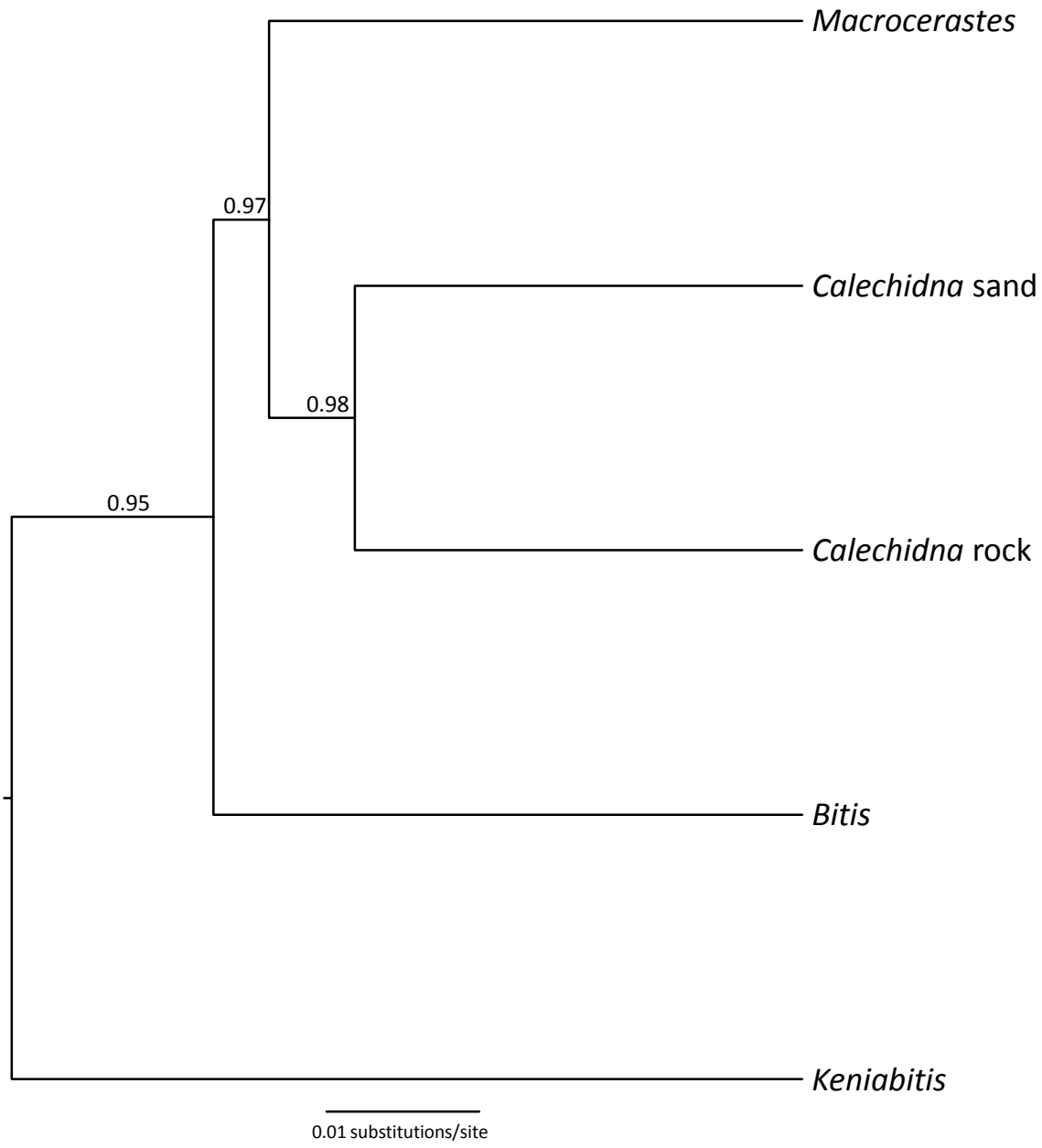


Figure S11. Subgenus level MSC species tree from the four locus analysis. Posterior probabilities are given at each node. Scale bar indicates substitutions/site.

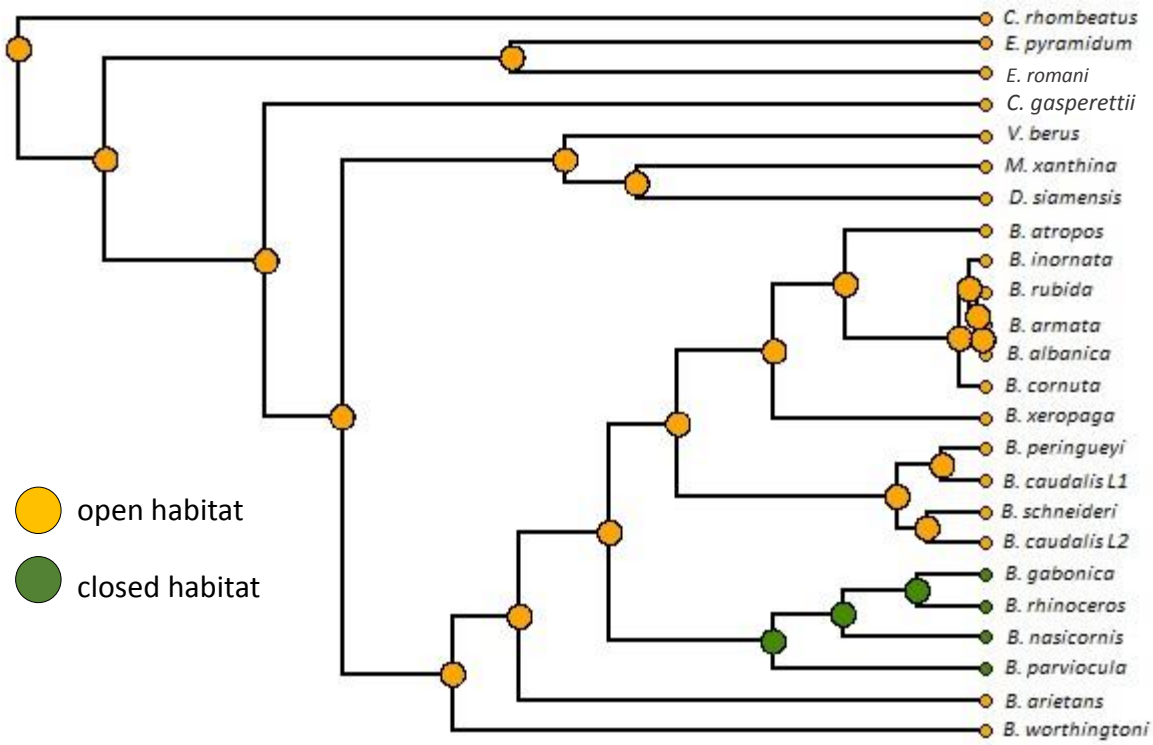


Figure S12. Character state coding and output from ancestral characters state optimisation for habitat type. Coloured circles at each terminal taxon represent the input character state coding. Coloured circles at each node indicate the estimated state for that node. All nodes were unequivocal based on their proportional likelihood scores (>0.99).

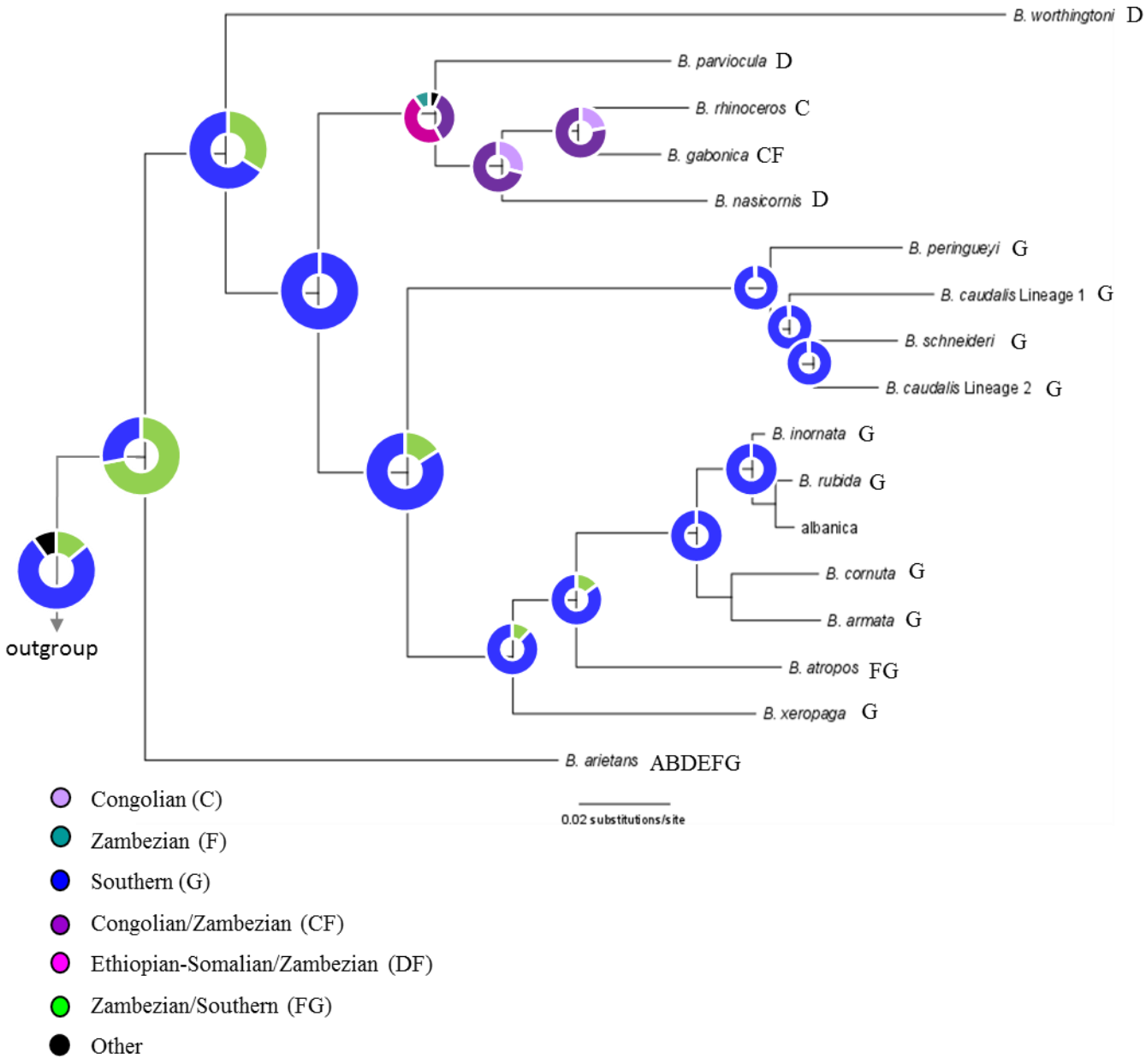


Figure S13. Character state coding for each tip and results of ancestral character state optimisation for area using the maximum likelihood phylogeny. Colours indicated at each node show the proportional likelihoods for area.

Figure S14. Map of southern Africa with place names indicated.

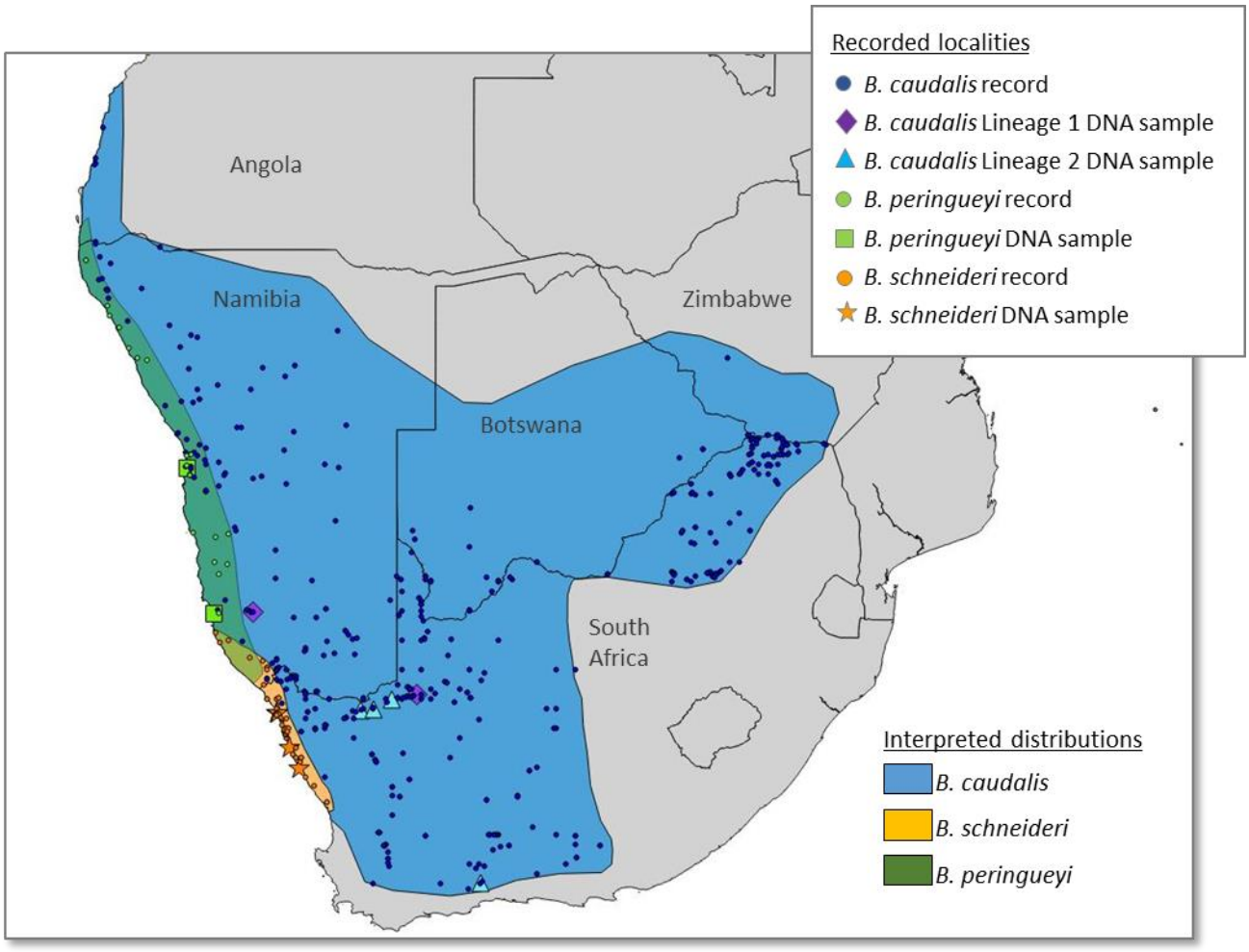


Figure S15. Interpreted distributions of *Bitis caudalis* sensu lato, *B. peringueyi* and *B. schneideri* shown as coloured polygons. Localities for each are also shown, as are the localities from which DNA samples were used for the present study. Samples for the two B. caudalis lineages are indicated by the purple diamond (Lineage 1) and light blue triangle (Lineage 2). Locality data are from Bates *et al.* 2014 and the Global Bioinformatics Information Facility (https://www.gbif.org/).

Table S1. Details of individuals sequenced for this study with corresponding GenBank accession numbers. Those indicated with an asterisk were downloaded from GenBank. NA - no data or information.

Species	**Sample**	**Locality**	**Country**	**16S**	**ND2**	**PRLR**	**UBN1**
Dabioa siamensis	AM-A2	NA	Thailand	MK387685	MK387481	MK387618	MK387557
Dabioa siamensis	AM-A22	NA	Myanmar	MK387686	MK387482	MK387619	MK387558
B. inornata	Bit10	Compassberg, Eastern Cape	South Africa	MK387660	MK387457	MK387596	MK387528
B. albanica	Bit11	Grassyridge, near Port Elizabeth, Eastern Cape	South Africa	MK387627	MK387426	NA	MK387491
B. rubida	Bit13	Ceres, Western Cape	South Africa	MK387670	MK387467	MK387606	MK387539
B. cornuta	Bit15	Carolusberg, Northern Cape	South Africa	MK387649	MK387447	MK387588	MK387517
B. caudalis	Bit16	32 km West of Pofadder, Northern Cape	South Africa	MK387643	MK387442	MK387583	MK387511
B. peringueyi	Bit20	Walvis Bay	Namibia	MK387667	MK387463	MK387602	MK387535
B. schneideri	Bit21	Port Nolloth, Northern Cape	South Africa	MK387673	MK387470	MK387608	MK387543
B. cornuta	Bit3	Carolusberg, Northern Cape	South Africa	MK387650	MK387448	NA	MK387518
B. cornuta	Bit4	Springbok, Northern Cape	South Africa	MK387651	MK387449	MK387589	MK387519
B. schneideri	Bit9	Port Nolloth, Northern Cape	South Africa	MK387674	MK387471	MK387609	MK387544
B. atropos	CMRK217	Nyanga National Park	Zimbabwe	MK387639	MK387438	MK387579	MK387506
B. worthingtoni	CMRK375	Near Timboroa, Rift Highlands	Kenya	MK387677	MK387473	MK387611	MK387547
B. caudalis	PEM28	Klein Aus	Namibia	MK387644	MK387443	MK387584	MK387512
B. arietans	WW1202	NA	Ghana	MK387629	MK387428	MK387569	MK387493
B. nasicornis	WW1241	Uganda	Uganda	MK387661	MK387458	MK387597	MK387529
B. nasicornis	WW1286	Ghana	Ghana	MK387662	MK387459	MK387598	MK387530
B. rhinoceros	WW1287	NA	Ghana	*EU624285	MK387466	MK387605	MK387538
Montivipera xanthina	WW1351	NA	NA	MK387689	MK387486	MK387624	MK387563
B. worthingtoni	WW1369	NA	Kenya	MK387678	MK387474	MK387612	MK387548
Montivipera xanthina	WW1374	NA	NA	MK387690	MK387487	MK387625	MK387564
B. cornuta	WW1379	Lüderitz	Namibia	MK387652	MK387450	MK387590	MK387520
B. xeropaga	WW1380	NA	NA	*EU624287	MK387476	MK387614	MK387551
B. rubida	WW1397	80km North of Ceres, Western Cape	South Africa	*EU624286	MK387468	MK387607	MK387540

Species	Sample	Locality	Country	16S	ND2	PRLR	UBN1
B. arietans	WW1409	Dodoma	Tanzania	MK387630	MK387429	MK387570	MK387494
B. atropos	WW1445	De Vlugt, Uniondale, Western Cape	South Africa	MK387640	*JX073287	*JX073298	MK387507
B. atropos	WW1446	Bettys Bay, Western Cape	South Africa	*EU624281	MK387439	MK387580	MK387508
B. rubida	WW1447	Swartberg, Western Cape	South Africa	MK387671	MK387469	NA	MK387541
B. arietans	WW1458	De Hoop, Western Cape	South Africa	MK387631	MK387430	MK387571	MK387495
Echis pyramidum	WW1521	Baringo	Kenya	NA	na	MK387620	MK387559
Echis pyramidum	WW1523	NA	Kenya	NA	MK387483	MK387621	MK387560
B. cornuta	WW1554	Springbok, Northern Cape	South Africa	MK387653	MK387451	MK387591	MK387521
B. xeropaga	WW1556	Vioolsdrif, Northern Cape	South Africa	MK387681	MK387477	NA	MK387552
B. arietans	WW1571	Agadir	Morocco	*EU624280	*JX073288	*JX073299	MK387496
B. arietans	WW1577	10 km N Calitzdorp, Western Cape	South Africa	*GQ359736	*JX073289	*JX073300	MK387497
Echis romani	WW1629	Garoua, Cameroon	Cameroon	MK387687	MK387484	MK387622	MK387561
Echis romani	WW1631	Garoua, Cameroon	Cameroon	MK387688	MK387485	MK387623	MK387562
B. arietans	WW1656	Bulawayo	Zimbabwe	MK387632	MK387431	MK387572	MK387498
B. arietans	WW1696	Wadi Darbat, Dhofar	Oman	*GQ359738	MK387432	MK387573	MK387499
B. atropos	WW1711	Swartberg, Western Cape	South Africa	MK387641	MK387440	MK387581	MK387509
B. rubida	WW1712	Swartberg, Western Cape	South Africa	MK387672	*JX073290	*JX073301	MK387542
B. armata	WW1728	De Hoop, Western Cape	South Africa	MK387635	MK387435	MK387576	MK387502
B. armata	WW1729	De Hoop, Western Cape	South Africa	MK387636	*JX073291	*JX073302	MK387503
B. caudalis	WW1752	Oudtshoorn, Western Cape	South Africa	MK387645	MK387444	MK387585	MK387513
B. armata	WW1754	De Hoop, Western Cape	South Africa	MK387637	MK387436	MK387577	MK387504
B. atropos	WW1762	Ladismith, Western Cape	South Africa	MK387642	MK387441	MK387582	MK387510
B. armata	WW1856	De Hoop, Western Cape	South Africa	MK387638	MK387437	MK387578	MK387505
B. gabonica	WW1873	Lobeke	Cameroon	MK387655	MK387453	MK387592	MK387523
B. nasicornis	WW1874	Lobeke	Cameroon	MK387663	MK387460	MK387599	MK387531
B. arietans	WW1890	Kasane	Botswana	MK387633	MK387433	MK387574	MK387500
B. gabonica	WW1919	Dukuduku, KwaZulu-Natal	South Africa	MK387656	MK387454	MK387593	MK387524
B. gabonica	WW1920	St. Lucia, KwaZulu-Natal	South Africa	MK387657	MK387455	MK387594	MK387525

Species	Sample	Locality	Country	16S	ND2	PRLR	UBN1
B. arietans	WW1940	60 km N Bambari	Central African Republic	MK387634	MK387434	MK387575	MK387501
B. albanica	WW1982	Grassyridge, near Port Elizabeth, Eastern Cape	South Africa	MK387628	MK387427	MK387568	MK387492
Vipera berus	WW199	Ynys Môn, Wales	United Kingdom	NA	MK387488	MK387626	MK387565
B. gabonica	WW2316	Tanga	Tanzania	MK387658	MK387456	MK387595	MK387526
Causus rhombeatus	WW2367	Plettenberg Bay, Western Cape	South Africa	MK387683	MK387478	MK387615	MK387554
B. caudalis	WW2443	Between Pofadder and Kakamas, Northern Cape	South Africa	MK387646	MK387445	MK387586	MK387514
B. caudalis	WW2445	Pofadder, Northern Cape	South Africa	MK387647	*JX073293	*JX073304	MK387515
B. caudalis	WW2448	Kakamas, Northern Cape	South Africa	MK387648	MK387446	MK387587	MK387516
B. xeropaga	WW2621	Aggeneys, Northern Cape	South Africa	MK387682	*JX073294	*JX073305	MK387553
B. worthingtoni	WW2624	NA	Kenya	MK387679	MK387475	MK387613	MK387549
B. worthingtoni	WW2625	NA	Kenya	MK387680	*JX073295	*JX073306	MK387550
B. cornuta	WW2650	Alexander Bay, Northern Cape	South Africa	MK387654	MK387452	NA	MK387522
B. gabonica	WW2714	Ganganya Brousse, Likouala	Republic of the Congo	MK387659	*JX073296	*JX073307	MK387527
B. nasicornis	WW2715	Impongui, Likouala	Republic of the Congo	MK387664	MK387461	MK387600	MK387532
B. schneideri	WW2811	Koringkorelbaai, Northern Cape	South Africa	MK387675	*JX073297	*JX073308	MK387545
B. schneideri	WW2812	Noup, Northern Cape	South Africa	MK387676	MK387472	MK387610	MK387546
B. peringueyi	WW2817	Kolmanskop	Namibia	MK387668	MK387464	MK387603	MK387536
B. parviocula	WW2980	Ethiopian Highlands	Ethiopia	MK387665	*JX073292	*JX073303	MK387533
B. parviocula	WW2981	Ethiopian Highlands	Ethiopia	MK387666	MK387462	MK387601	MK387534
Cerastes gasperettii	WW3170	NA	Saudi Arabia	NA	MK387479	MK387616	MK387555
Vipera berus	WW3252	Gwynedd, Wales	United Kingdom	MK387691	MK387489	NA	MK387566
Cerastes gasperettii	WW3257	NA	Saudi Arabia	MK387684	MK387480	MK387617	MK387556
B. rhinoceros	WW3376	NA	NA	MK387669	MK387465	MK387604	MK387537
Vipera berus	WW3384	Jyväskylä	Finland	MK387692	MK387490	NA	MK387567

Table S2. Ancestral area coding using biogeographic regions from Fig. S1.

	Taxon coding	Area
1	*Causus rhombeatus*	CDEFG
2	*Cerastes gasperettii*	ABE
3	*Daboia siamensis*	AB
4	*Echis romani*	ADE
5	*Echis pyramidum*	ADE
6	*Montivipera xanthina*	AB
7	*Vipera berus*	A
8	*Bitis albanica*	G
9	*Bitis arietans*	ABDEFG
10	*Bitis armata*	G
11	*Bitis atropos*	FG
12	*Bitis caudalis L1*	G
13	*Bitis caudalis L2*	G
14	*Bitis cornuta*	G
15	*Bitis gabonica*	CF
16	*Bitis inornata*	G
17	*Bitis nasicornis*	C
18	*Bitis parviocula*	D
19	*Bitis peringueyi*	G
20	*Bitis rhinoceros*	C
21	*Bitis rubida*	G
22	*Bitis schneideri*	G
23	*Bitis worthingtoni*	D
24	*Bitis xeropaga*	G

Area coding key	
A	Eurasia
B	North Africa inc. Saharan
C	Congolian
D	Ethiopian/Somalian
E	Sudanian
F	Zambezian
G	Southern

Table S3. Range constraints included in estimations for ancestral areas with the DEC analysis.‘x’ indicates that a particular range would be included as possible. e.g. an 'x' in row A and column B indicates the range AB was included..

		Eurasia	North Africa	Congolian	Ethiopian/Somalian	Sudanian	Zambezian	Southern
		A	B	C	D	E	F	G
Eurasia	A		x		x			
North Africa	B			x	x	x		
Congolian	C				x	x	x	
Ethiopian/Somalian	D					x	x	
Sudanian	E						x	
Zambezian	F							x
Southern	G							

Table S4. Relative probabilities of dispersal between areas at four time periods used in the DEC analysis.

0-2myr		Eurasia	North Africa	Congolian	Ethiopian/Somalian	Sudanian	Zambezian	Southern
		A	B	C	D	E	F	G
Eurasia	A	1	0.25	0	0.25	0	0	0
North Africa	B	0.25	1	0	0.25	1	0.25	0
Congolian	C	0	0	1	0.25	0.25	0.25	0
Ethiopian/Somalian	D	0.25	0.25	0.25	1	1	1	0
Sudanian	E	0	1	0.25	1	1	0.25	0
Zambezian	F	0	0.25	0.25	1	0.25	1	1
Southern	G	0	0	0	0	0	1	1

2-11myr		Eurasia	North Africa	Congolian	Ethiopian/Somalian	Sudanian	Zambezian	Southern
		A	B	C	D	E	F	G
Eurasia	A	1	0.25	0	0.25	0	0	0
North Africa	B	0.25	1	0.25	1	1	0.25	0
Congolian	C	0	0.25	1	0.25	0.5	1	0
Ethiopian/Somalian	D	0.25	1	0.25	1	1	1	0
Sudanian	E	0	1	0.5	1	1	0.5	0
Zambezian	F	0	0.25	1	1	0.5	1	1
Southern	G	0	0	0	0	0	1	1

11-30myr		Eurasia	North Africa	Congolian	Ethiopian/Somalian	Sudanian	Zambezian	Southern
		A	B	C	D	E	F	G
Eurasia	A	1	0.1	0	0.25	0	0	0
North Africa	B	0.1	1	0.5	0.5	1	0.25	0
Congolian	C	0	0.5	1	1	1	1	0
Ethiopian/Somalian	D	0.25	0.5	1	1	1	1	0

Sudanian	E	0	1	1	1	1	0.5	0
Zambezian	F	0	0.25	1	1	0.5	1	1
Southern	G	0	0	0	0	0	1	1

47myr		**Eurasia**	**North Africa**	**Congolian**	**Ethiopian/Somalian**	**Sudanian**	**Zambezian**	Southern
		A	**B**	**C**	**D**	**E**	**F**	**G**
Eurasia	A	1	0.1	0	0.1	0	0	0
North Africa	B	0.1	1	1	1	1	0.25	0
Congolian	C	0	1	1	1	1	1	0
Ethiopian/Somalian	D	0.1	1	1	1	1	1	0
Sudanian	E	0	1	1	1	1	0.75	0
Zambezian	F	0	0.25	1	1	0.75	1	1
Southern	G	0	0	0	0	0	1	1

Table S5. Probabilities for key nodes in the Bitis phylogeny estimated by *BEAST (a) and maximum likelihood (b). Highest probabilities are in bold red font, and those that match between analyses are highlighted in yellow. The area coding used is left top. The node numbers differ between topologies and are given to the right of each of the probability matrices.

ANCESTRAL STATE PROBABILITIES: DEC ANALYSIS WITH *BEAST TOPOLOGY															
AREA/NODE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C	0	0	0	0	0	0	0	0	0	0	0.29	0.23	0	0	0
CD	0	0	0	0	0	0	0	0	0	0.08	0	0	0	0	0
CF	0	0	0	0	0	0	0	0	0	**0.32**	**0.71**	**0.77**	0.01	0.01	0.01
DF	**1.00**	**0.57**	0	0	0	0	0	0	0	**0.43**	0	0	0	0	0
F	0	0.22	0.23	0	0	0	0	0	0	0.17	0	0	0	0	0
FG	0	0.21	**0.67**	**1.00**	0.18	0.13	0.20	0	0	0	0	0	0	0	0
G	0	0	0.10	0	**0.82**	**0.88**	**0.80**	**1.00**	**1.00**	0	0	0	**0.99**	**0.99**	**0.99**

Node description	
1	origin of Bitis (divergence from outgroup)
2	divergence of B. worthingtoni
3	divergence of B. arietans
4	Split between Macrocerastes and Calechidna clades
5	Split between rupicolus and arenicolus Calechidna clades
6	divergence of B. xeropaga
7	divergence of B. atropos
8	divergence of B. cornuta
9	divergence of B. inornata
10	divergence of B. parviocula
11	divergence of B. nasicornis
12	divergence of B. rhinoceros with B. gabonicus
13	divergence between B. schneideri and B. caudalis L2
14	divergence of B. pernigueyi and B. caudalis L1
15	divergence between B. schneideri+B. caudalis L2 and B. peringueyi+B. caudalis L1

b) ANCESTRAL STATE PROBABILITIES: DEC ANALYSIS WITH MAXIMUM LIKELIHOOD TOPOLOGY															
AREA/NODE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C	0	0	0	0	0	0	0	0	0	0	0.29	0.22	0	0	0
CD	0	0	0	0	0	0	0	0	0	0.07	0.00	0.00	0	0	0
CF	0	0	0	0	0	0	0	0	0	**0.35**	**0.71**	**0.78**	0	0	0
DF	0	0	0	0	0	0	0	0	0	**0.48**	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0.10	0	0	0	0	0
FG	0.14	**0.72**	**0.66**	**1.00**	0.16	0.12	0.15	0	0	0	0	0	0	0	0
G	**0.76**	0.28	0	0	**0.84**	**0.88**	**0.85**	**1.00**	**1.00**	0	0	0	**0.99**	**0.99**	**0.99**
OTHER	0.10	0	0	0	0	0	0	0	0	0	0	0	0.01	0.01	0.01

Node description	
1	origin of Bitis (divergence from outgroup)
2	divergence of B. arietans
3	divergence of B. worthingtoni
4	Split between Macrocerastes and Calechidna clades
5	Split between rupicolus and arenicolus Calechidna clades
6	divergence of B. xeropaga
7	divergence of B. atropos
8	divergence of B. cornuta+armata
9	divergence of B. inornata
10	divergence of B. parviocula
11	divergence of B. nasicornis
12	divergence of B. rhinoceros with B. gabonicus
13	divergence of B. peringueyi
14	divergence of B. caudalis L1
15	divergence between B. schneideri and B. caudalis L2

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Ancient habitat shifts and organismal diversification are decoupled in the African viper genus *Bitis* (Serpentes: Viperidae).

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Running Title: Diversification in vipers (*Bitis*)

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ABSTRACT

Aim The expansion of open habitats during the mid-Miocene has been hypothesised as a driver of allopatric speciation for many African taxa. This habitat-dependent mode of diversification has been implicated in the shift from C_3 (e.g. forest/woodland) to C_4 dominated systems (i.e. open savanna, grasslands) in a number of African squamates. We examined this hypothesis using a genus of African viperid snakes (*Bitis*) with both open habitat and forest-dwelling representatives.

Location Africa.

Methods A comprehensive multilocus dataset was used to generate a calibrated species tree using a multispecies coalescent model. Individual gene trees and patterns of nuclear allele sharing were used to assess species monophyly and isolation. To test the habitat-dependent evolution hypothesis, we generated an ancestral character state reconstruction for open and closed habitats using the dated phylogeny. This was related to the timing of open habitat expansion and forest/woodland contraction in Africa.

Results The genus *Bitis* originated in the Oligocene, with species level diversification in the late Miocene/Pliocene. Four well-supported clades correspond to the recognised subgenera *Bitis, Keniabitis, Macrocerastes* and *Calechidna*. Several previously unrecognised lineages potentially represent cryptic species.

Main Conclusions Habitat-dependent evolution does not appear to have been a main driver for generic level viperine diversification: the ancestral state for *Bitis* was open habitat and at least one clade moved into forest in the Miocene, long after forest had contracted and fragmented. Forest dependent species diversified only in the late Miocene, presumably as forest became further reduced in extent, fitting an allopatric model of speciation. Although our results do not favour a general pattern of habitat-dependent diversification in *Bitis*, cladogenesis within the subgenus *Calechidna* for 'arenicolous' species (*B. caudalis* complex) and 'rupicolous' species (*B. atropos-cornuta* complex), corresponds to the aridification of southwest Africa. This suggests there are subtleties not captured in the broad open habitat category, which are relevant for understanding the role of habitat-dependent evolution.

Keywords

sub-Saharan Africa, multilocus phylogenetics, multispecies coalescent, reptiles, snakes

INTRODUCTION

In broad terms, sub-Saharan African faunal lineages can be segregated into those that occupy closed or dense canopy~~, tropical rainforest~~ forest/woodland ecosystems ~~((~~"forest" lineages) and those that occupy structurally more open ecosystems such as ~~woodland,~~ grassland, heathlands, open savanna and desert ~~((~~"open ~~-~~habitat" lineages: e.g. deMenocal, 1995, 2004; Tolley *et al*., 2008, 2011; Maslin *et al*., 2014). ~~Until~~Through the ~~mid-Eocene,~~ Paleogene (66-23 Mya) dense woodland/forest ~~ecosystems~~ were ~~the dominant vegetation type~~widespread across sub-Saharan Africa, ~~which was~~and were gradually displaced by open ecosystems through the Oligocene and early Miocene as the tropical climate aridified (Coetzee, 1993; Morley, 2007; Kissling *et al*., 2012; Linder 2017). During the Oligocene, forest/woodland became reduced in extent, contracting from North Africa and the Southern & Zambezian region into Central Africa presumably leaving ~~a~~ substantial patches in central ~~belt of forest~~Africa (see Morley, 2007; Figs S1 & S2 in Supporting Information), possibly as a mosaic with more open vegetation types (Linder, 2017). From the mid to late Miocene, beginning ca. 10 million years ago (mya), open habitats expanded markedly, with those comprised primarily of plant species utilising the C_4 photosynthetic pathway becoming increasingly dominant on the continent (Couvreur *et al*., 2008; Edwards *et al*., 2010; Kissling *et al*., 2012; Maslin *et al*., 2014~~.~~). Subsequent climatic cooling and aridification during the Pliocene and Pleistocene, 2.8–1.0 mya, was associated with further open habitat expansion and the dominance of C_4 grasslands and savanna (deMenocal, 1995; Kissling *et al*., 2012). This aridification was punctuated by short moist periods that could have facilitated temporary forest re-expansion (Trauth *et al*., 2005; Maslin *et al*., 2014). Regardless, since the Cretaceous, ~~Africa appears to have~~the widespread forest/woodland lost ~~as much as 85%~~most of its ~~forest~~ extent, with open habitats becoming dominant in the landscape (Morley, 2007; Kissling *et al*., 2012).

The prominent expansion of open habitats in sub-Saharan Africa is thought to have played a key role in the evolution of open habitat fauna. Multiple hypotheses have been invoked to explain this faunal evolution in open habitats (Vrba, 1985, 1992; Potts, 1998), and collectively these have been termed "habitat-specific hypotheses" (Potts, 1998; deMenocal, 2004). The paradigm essentially points to ecological speciation, where diversification is driven by directional selection in differing environments (e.g. Rundil & Nosil, 2005; Schluter, 2009). Here, we adopt the term 'habitat-dependent' evolution to specifically refer to ecological diversification of lineages inhabiting novel habitats due to reorganisation of habitat types on the African continent.

The mammalian fossil record provides considerable evidence for habitat-dependent evolution in sub-Saharan Africa. In particular, the expansion of C_4 grassland during the Plio-Pleistocene appears to have played a role (Hewitt, 2004) as the first appearance of many arid adapted species across a range of taxa coincides with this period (Wesselman, 1985; Vrba, 1992; Bobe *et al*., 2002; Bobe & Behrensmeyer, 2004; Bowie & Fjeldså, 2008). Phylogenetic studies also support this hypothesis, with a number of forest dependent taxa showing strong signatures of allopatric speciation corresponding to fragmentation of forests (Bowie *et al*., 2006; Tolley *et al*., 2008; Lawson, 2010; Demos *et al*., 2014; Menegon *et al*., 2014; Barej *et al*., 2015), whereas recent radiations appear to correspond with occupation of more open habitats (Tolley *et al*., 2006; Bowie & Fjeldså, 2008; Tolley *et al*., 2013; Demos *et al*., 2014). These patterns are clearly taxon dependent, presumably because of the

idiosyncratic life-history characteristics and dispersal ability of the taxa. In general however, highly vagile species are either generalists, or can disperse across unsuitable habitat (Oatley *et al*., 2012; Fuchs *et al*., 2013), which facilitates gene flow resulting in low genetic structure. In contrast, most forest dependent species will find the open habitat a formidable barrier and require either forest reconnection or habitat corridors to maintain population connectivity and gene flow (Bowie *et al*., 2006; Measey & Tolley, 2011; Barej *et al.*, 2015; Bittencourt-Silva *et al*., 2016). Given taxon idiosyncrasies, a universal model for the evolution of fauna on the continent is not plausible. However, a paradigm that incorporates the reduction of forest/woodland as an important driver of biogeographic patterns is tenable and can incorporate the idiosyncratic nature of species.

Squamate reptiles are a taxonomic group that is both widespread and highly diverse within sub-Saharan Africa, where diversification of forest/woodland dependent taxa has been influenced by habitat shifts. For example, several clades of squamates that currently occupy open habitats diversified within the Miocene (e.g. chameleons and snakes; Wüster *et al*., 2007; Pook *et al*., 2009; Barlow *et al.*, 2013; Tolley *et al.*, 2013). Furthermore, ancient forest lineages in the southern African chameleon genus *Bradypodion* gave rise to open-habitat species following the onset of open habitat expansion in the Pliocene (Tolley *et al*., 2008; Measey *et al*., 2009; Edwards *et al*., 2012; da Silva *et al*., 2014; da Silva & Tolley, 2017), suggesting that shifts to open habitats beginning in the Miocene may have been widespread on the landscape and across multiple taxonomic groups.

The African viper genus *Bitis* provides an opportunity to test the habitat-dependent hypothesis of ecological diversification. Commonly referred to as the African adders, *Bitis* is Africa's most taxonomically diverse and geographically widespread viperid genus, containing eighteen extant species (*sensu* Lenk *et al*., 1999; Branch, 1999, Gower *et al*., 2016; Uetz *et al*., 2017) and one documented extinct Pleistocene species, *Bitis olduvaiensis* (Rage, 1973). Several studies have investigated the phylogeny of *Bitis* using morphological evidence (Groombridge, 1980; Ashe & Marx, 1988; Wittenberg *et al*., 2015) and immunological distances (Lenk *et al.*, 1999). Higher level phylogenies of Viperidae and Viperinae have also included *Bitis* (Herrmann & Joger, 1995, 1997; Herrmann *et al*., 1999; Lenk *et al.*, 1999, 2001; Wüster *et al.*, 2008; Pyron *et al.*, 2013; Alencar *et al.*, 2016). The study of Lenk *et al.* (1999) identified four major mitochondrial clades within the genus *Bitis*, which were formally recognised as subgenera (Table 1). These are:

- *Macrocerastes*, a clade of large-bodied forest adders, which includes the Gaboon adders (*B. gabonica* and *B. rhinoceros*) and the Rhinoceros viper (*B. nasicornis*).
- *Calechidna*, a clade of open habitat dwarf adders endemic to southern Africa. This clade is further divided into two subclades corresponding, respectively, to those taxa primarily associated with gravel or rocky habitats ("rupicolous", *B. atropos-cornuta* complex) and those associated with sandy substrates ("arenicolous", *B. caudalis* complex).
- *Keniabitis*, a monotypic clade representing the small-bodied Kenyan endemic *B. worthingtoni*, which occurs in montane grassland habitats along the Kenyan Rift Valley.
- *Bitis* (the type subgenus), representing the geographically widespread and large-bodied puff adder (*B. arietans*), which occurs across a variety of open woodland, grassland and scrubland habitats throughout sub-Saharan Africa, southern Arabia and Morocco.

Although the evolutionary relationships within *Bitis* are relatively well understood, several important questions remain. The relationship between the subgenera lacks resolution, and the phylogenetic positions of *B. (K.) worthingtoni* and *B. (B.) arietans* were equivocal in previous analyses due to a lack of statistical support at basal nodes (Lenk *et al*., 1999; Wüster *et al*., 2008; Pyron *et al*., 2013). In addition, several poorly known species have not been included in any molecular phylogeny to date (*B. harenna*, *B. albanica*, *B. heraldica* and *B. inornata*), and most studies of *Bitis* have utilised single individuals to represent species, precluding any assessment of levels of intraspecific genetic diversity or the testing of species monophyly (but see Barlow *et al*., 2013).

In this study, we examine evolutionary relationships within *Bitis* to investigate whether a habitat-dependent hypothesis of diversification applies to this genus. We used a time-calibrated multilocus phylogeny, including 16 of the 18 currently recognised *Bitis* species, to explore patterns and timing of diversification among the subgeneric clades. In particular, we expected that *Bitis* lineages occupying open habitats (subgenera: *Calechidna*, *Keniabitis* and *Bitis*) diverged either in response to the initial but gradual aridification of Africa (Eocene/Oligocene) or later, during the rapid mid-Miocene expansion of open habitats. If so, the origin of the genus should reflect the geographic region where the forest/woodland contraction was maximal during those time periods (either North Africa or the Southern & Zambezian regions). We carried out ancestral character state reconstruction of the broad habitat categories (forest/woodland mosaic and open-habitat), to understand if the timing of diversification corresponded to major habitat shifts on the continent, which could support habitat-dependent diversification. Furthermore, an ancestral area reconstruction allowed us to assess whether the geographic origin of key clades fits well with habitat-dependent diversification. We also included multiple representatives of species to investigate the outstanding taxonomic issues, specifically subgeneric and species monophyly and the possibility of cryptic speciation.

MATERIAL AND METHODS

Tissues (scale clips, blood, shed skins, dermal tissue or liver) were sampled from all currently recognised *Bitis* species except the poorly known Angolan species *B. heraldica* and the recently described *B. harenna*. All individuals were released after sampling or retained alive by their owners. Multiple representatives of each sampled species were included except for *B. inornata* and *B. rhinoceros*, for which it was only possible to sample a single individual. Sequences from additional representatives of the Viperidae were also generated or downloaded from GenBank for use as outgroup taxa and to facilitate the dating analysis. Outgroup taxa included one to three individuals from six other genera (from Africa and Eurasia) in the subfamily Viperinae, resulting in a dataset of 77 individuals for 4 genes. Of these, sequences of one to three genes from 15 individuals were available on GenBank. Details of samples, vouchers and GenBank accession numbers are given in Table S1 in Supporting Information.

We generated sequence data from two mitochondrial and two unlinked nuclear markers. The mitochondrial data consisted of partial sequences of the 16s ribosomal RNA (16s) and NADH dehydrogenase subunit 2 (ND2) genes. The nuclear markers were exonic sequences of the prolactin receptor (PRLR) and ubinuclein 1 (UBN1) genes. Total DNA was extracted

from tissue samples using a Qiagen DNeasy™ Tissue Kit (cat. no. 69506) following the manufacturer's instructions. Genetic markers were PCR amplified using the following primers. 16s: L2510 (5'-CGCCTGTTTATCAAAAACAT-3') and H3080 (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.*, 1991); ND2: L4437b (5'-CAGCTAAAAAAGCTATCGGGCCCATAC-3') (Kumazawa *et al.,* 1996) and tRNA-trpR (5'-GGCTTTGAAGGCTMCTAGTTT-3') (Ashton & de Queiroz, 2001); PRLR: PRLR-f1 (5'-GACARYGARGACCAGCAACTRATGCC-3') and PRLR-r3 (5'-GACYTTGTGRACTTCYACRTAATCCAT-3') (Townsend *et al*., 2008); UBN1: BaUBN_F (5'-CCTCTGGTTACTCAGCAGCA-3') and BaUBN_R (5'-ATTGGCCACTCCTTGTGTTC-3'). PCRs comprised 9.6 µl ABgene ReddyMix™ PCR Master Mix (cat. no. AB-0575/LD/A), 0.27 µM of each primer and 5–10 ng of template DNA, giving a final reaction volume of 11 µl. The thermocycling regimes involved an initial denaturation at 94°C for 2 min; 30–40 cycles of: 30 s denaturation at 94°C, 30 s (16s, ND2) or 60 s (PRLR, UBN1) annealing at 50°C (16s), 52°C (PRLR), 55°C (ND2), or 60°C (UBN1), and 45 s (16s, PRLR, UBN1) or 90 s (ND2) extension at 72°C; and a final extension for 5 min at 72°C. PCR products were cleaned using the enzymes exonuclease 1 and thermo-sensitive alkaline phosphatase, and direct sequencing carried out by Macrogen Inc. (dna.macrogen.com) using forward PCR primers (16s, some PRLR) or both forward and reverse PCR primers (ND2, UBN1, some PRLR).

Sequences were proof-read and aligned using the software CodonCode Aligner ~~v.~~ 3.5.6 (www.codoncode.com). Only clean sequences were retained, and we re-sequenced any sequence with questionable stretches. Protein-coding gene sequences were translated to check that no frameshift mutations or stop codons were present. Alignment was ambiguous for some sections of the 16s alignment so these regions were excluded from analyses. UBN1 contained a 'TCC' tri-nucleotide repeat section with several heterozygous indels necessitating the exclusion of 30 bp.

Heterozygous positions were identified in nuclear sequence chromatograms by a combination of visual inspection for double peaks and typically low quality Phred scores (Ewing & Green, 1998) for the bases surrounding a heterozygous position. Individual allele sequences were estimated from the diploid nuclear sequences using PHASE (Stephens *et al.,* 2001; Stephens & Scheet, 2005) in DnaSP v. 5 (Librado & Rozas, 2009), using default settings. To verify the reliability of the PHASE, analysis we computed maximum likelihood trees under the GTRCAT model in RAxML 7.2.8 (Stamatakis, 2006) for both the unphased and phased alignments, with clade support assessed using 100 bootstrap replicates and specifying the *Causus* sequences as outgroup. For each nuclear gene, both phased and unphased alignments produced highly congruent topologies with broadly comparable bootstrap values for all nodes above the species level (Supporting Information Figs S3-6). Overall, this indicates no obvious distortion of phylogenetic signal in either dataset as a result of the phasing procedure. The final dataset consisted of 2415 base pairs: 16S-426bp; ND2-1014bp; PRLR-525bp; UBN1-450bp.

Species relationships were first investigated by concatenating data from all loci. A maximum likelihood (ML) search was run using RAxML HPC ~~v7~~7.2.8 (Stamatakis, 2006) on the CIPRES Science Gateway (~~www.phylo.org/sub_sections/portal/)~~Miller et al. 2010) for the 4-gene dataset. The analysis was run using both unphased and phased nuclear sequences. Each gene was partitioned separately, and the default GTR+I+G model was used with rapid

bootstrapping halted automatically (Stamatakis et al. 2008). This analysis was run three times to ensure that independent ML searches produced the same topologies. We considered nodes with a bootstrap value of >70% as supported in this analysis.

The *Bitis* species tree was then inferred using a multispecies coalescent (MSC) model using *BEAST (Heled & Drummond, 2010), implemented in BEAST v. 1.7.4 (Drummond *et al*., 2012). *BEAST co-estimates individual gene trees and the species tree within which they evolved, using a fully Bayesian framework accounting for incomplete lineage sorting. We assigned individuals to species according to current taxonomy (Lenk *et al.*, 1999) except in the case of *B. caudalis*, which preliminary analysis found to comprise two polyphyletic mitochondrial lineages (see Results). Individuals corresponding to these mitochondrial lineages were therefore assigned as separate taxa (*B. caudalis* L1 and L2). Including outgroup taxa, the resulting species tree contained 24 species/taxa, sampling 77 individuals, and was inferred from three independent gene trees: mitochondrial (estimated from concatenated 16s and ND2 sequences), PRLR and UBN1.

We estimated timing of divergence among *Bitis* species by calibrating the MSC species tree analysis based on fossil evidence from the related Eurasian viperine clade (represented by *Vipera berus*, *Daboia siamensis* and *Montivipera xanthina*), which the fossil record shows to have existed at least 20 mya (Szyndlar & Rage, 1999). Based on the assumption that the most recent common ancestor (MRCA) of this clade is unlikely to have occurred considerably earlier than this, we constrained the monophyly of this clade and applied a lognormal prior to the age of the MRCA with a 20 mya offset, mean of zero and standard deviation of 1.0, and upper limit of 40 mya. Head *et al.* (2016) argued that while fossil vertebrae of the "*aspis* complex" of Szyndlar & Rage (1999) can be assigned to that lineage, other viperine vertebrae would be difficult to assign to any particular group of viperines, or even to distinguish from crotaline remains. They therefore suggested that this calibration point can only be used to date the divergence of viperines and crotalines. However, if the "*aspis* complex" fossils of Szyndlar & Rage (1999) can indeed be assigned to the genus *Vipera* based on apomorphies, then it logically follows that they can and should be used to calibrate the divergence of that genus from its sister group, most likely *Daboia* (Wüster *et al*., 2008; Pyron *et al.*, 2013; Alencar *et al*., 2016), not the older split between viperines and crotalines. Given the relative scarcity of early Miocene/Oligocene viperid fossils, we prefer a less narrowly constrained upper age limit for this calibration point than suggested by Head *et al.* (2016).

Separate, unlinked nucleotide substitution models were specified for each gene, selected from those available in BEAUTI under the Bayesian information criterion (BIC) in MEGA5 (Tamura *et al*., 2011). Uncorrelated, lognormal relaxed clock models were specified for each gene. A Yule speciation prior with piecewise linear population size model and constant root was specified for the species tree. The final analysis was carried out on Bioportal (www.bioportal.uio.no), and involved three independent runs of $5x10^8$ generations that sampled the ~~MCMC~~Markov chain Monte Carlo every 50,000 generations. The first 10% of samples from each run was removed as burn in. Convergence and adequate sampling (effective sample sizes > 200) of all parameters was verified in TRACER ~~v.~~ 1.5 (Rambaut & Drummond, 2007). The maximum clade credibility tree was selected from the combined posterior sample of 27,000 species trees and annotated with posterior clade probabilities

and node heights equal to the median value from the posterior sample using TreeAnnotator. We consider posterior probabilities ≥ 0.90 as providing moderate clade support, and those ≥0.95 as providing strong support.

We also examined the individual gene trees resulting from the *BEAST analysis, which are estimated independently of the species designations used to constrain the species tree. We checked whether current species designations correspond with monophyletic clades in the gene trees, and also looked for the existence of divergent genetic lineages within currently described species that may indicate the presence of monophyletic species complexes.

As the time taken for nuclear markers to reach reciprocal monophyly is expected to exceed that of mitochondrial markers due to an expected four-fold reduction in effective population size of the latter, we also investigated whether currently recognised species possess unique nuclear alleles. The presence of unique alleles provides evidence of lineage isolation because shared alleles are expected to be lost over time due to genetic drift, before reciprocal monophyly has been achieved. Shared alleles, in contrast, could indicate allele sharing between groups due to ongoing gene flow, or alternatively a relatively recent speciation event. The ability to detect shared alleles is governed by sample sizes, which are relatively small for the majority of species studied here. Nuclear allele sharing can thus only be seen as an additional line of evidence for lineage isolation, rather than as providing conclusive support.

As an independent indicator of relationships among subgenera, we included an additional nuclear marker, the anonymous nuclear marker Ba34 (Barlow *et al*., 2012). Ba34 sequences were not available for all species, precluding their use in the species-level *BEAST analysis. However, all four subgenera, including both sand- and rock-dwelling *Calechidna* clades, are represented by published sequences (Barlow *et al*., 2012). These were phased (as described previously) and analysed using *BEAST, assigning sequences to one of the five major *Bitis* clades. Relaxed clock models were used for data partitions and the HKY substitution model specified for Ba34. Other aspects of the analysis were as described previously.

Ancestral character state estimation for habitat was carried out using the APE 3 and Phytools packages in R (Paradis, 2012; Popescu *et al*., 2012; Revell, 2012). Each taxon was coded as occurring in closed (forest/woodland) or open (e.g. open savanna, karroid, grassland, heathland, desert) habitat (Fig. 1). Outgroup taxa were included to polarize the analysis, and were coded as belonging to open habitats (this being the dominant habitat across each outgroup genus included; Phelps, 2010). Because five Viperinae genera were missing from our analysis, we must treat the results of this analysis with caution. However, it should be noted that four of these five missing genera occur in open habitats, with only *Atheris* found in forest. A more comprehensive Viperinae phylogeny would be needed to test whether inclusion of *Atheris* and the other genera would change our results. The reconstructions were run with the 'ace' function using the equal states Markovian (Mk) model of character evolution (https://www.r-phylo.org/wiki). The ancestral habitat reconstruction analyses were also run in Mesquite ~~v.~~ 3.6 (Maddison & Maddison, 2018) using the same character coding, a likelihood optimization, and the Mk model. Because the ML topology differed from the MSC species tree in the positon of *B. arietans* and *B.*

worthingtoni, both of the ancestral habitat analyses were run on the maximum likelihood tree (pruned to retain one tip per taxon as in Fig. 1b) as well as on the MSC species tree.

An ancestral area reconstruction was carried out using a Dispersal-Extinction-Cladogenesis model (DEC; Ree & Smith, 2008) in RASP ~~v.~~ 4.0 beta (Yu *et al.*, 2015) using the ultrametric MSC species tree generated in *BEAST, and including the six outgroup genera from the Viperinae (*Causus, Cerastes, Daboia, Echis, Montivipera, Vipera*). The analysis was also run on the maximum likelihood tree (pruned to retain one tip per taxon as in Fig. 1b). The terminal taxa for *Bitis* were coded for the analysis based on their known distributions, whereas the taxa that represented the six Viperinae genera were coded according to the distribution of the entire genus (see Phelps, 2010). The following regions were used for the coding: Eurasia, North Africa (including Saharan), Sudanian, Congolian, Ethiopian, Somalian, Zambezian, Southern following the biogeographic regions from Linder *et al.* (2014; Fig. S2 & Table S2 in Supporting Information). The DEC analysis allows for both range and dispersal constraints to be defined, so that lineage dispersal can be modelled taking into account timing of divergences and the connectivity between geographic regions (Ree *et al.*, 2005). Ancestral ranges were constrained to adjoining geographic regions (Table S3 in Supporting Information). Dispersal probabilities between regions were assigned at four time points (0-2 mya, 2-11 mya, 11-30 mya, 30-47mya; Table S4 in Supporting Information) based on the potential for connectivity between regions. This was guided by present day vegetation and climate of the continent and paleo-vegetation maps for Africa (Morley, 2007; Kissling *et al.*, 2012; Fig. S1 in Supporting Information).

RESULTS

Both MSC species tree and ML analyses of the concatenated alignment supported the monophyly of *Bitis* and its subdivision into four previously recognised subgeneric clades (Fig 1, Figs S7 & S8 in Supporting Information). However, these methods supported different relationships between some major clades. The MSC species trees have *Keniabitis* (*Bitis worthingtoni*) sister to all other species of *Bitis*, and showed moderate support (0.90 pp) for *Bitis arietans* as sister to *Calechidna* + *Macrocerastes*. In contrast, the ML topology for the concatenated alignment shows *B. arietans* (100% bootstrap) as sister to all other species (Fig. 1b, Fig. S8 in Supporting Information). The topologies from the ML and MSC analyses for the four-gene dataset also differed slightly for some clades within the *Calechidna* (Fig. 1b), although the ML and mitochondrial gene tree generated in the MSC analysis were in agreement for these relationships (Fig. 2).

In other respects, topologies from the two methods (MSC and ML) were in agreement, and there were no discrepancies between the unphased (Fig. S8 in Supporting Information) and phased (figure not included) ML topologies. Furthermore, the *BEAST analysis supported monophyly of the four subgeneric clades for each individual gene tree (Figs S9-10 in Supporting Information), with the exception of *Calechidna*, for which monophyly was not supported in the PRLR and UBN1 trees. The position of *B. arietans* was sister to all other *Bitis* in the PRLR tree, albeit without notable support. The inclusion of sequences of the anonymous nuclear marker Ba34 provided improved resolution of relationships among the major clades (Fig. S11 in Supporting Information), providing strong support for the *Calechidna* + *Macrocerastes* + *B. arietans* clade (posterior probability 0.95 compared to 0.90 in the three locus analysis).

Relationships among the four representatives of the subgenus *Macrocerastes* are well resolved in the species and ML trees, with the two Gaboon adders (*B. rhinoceros* and *B. gabonica*) sister to each other. *Bitis nasicornis* forms the sister group to this Gaboon adder clade, with *B. parviocula* in turn sister to this clade (Fig. 1). Individual gene trees largely recovered identical relationships and the monophyly of all species was strongly supported with the exception of *B. nasicornis* in the UBN1 tree (Fig. S10 in Supporting Information). All recognised species exhibited unique alleles with the exception of *B. rhinoceros* and *B. gabonica*, which share PRLR alleles (Fig. 2b).

Species tree and ML analyses supported the subdivision of *Calechidna* into two clades corresponding to the rupicolous and arenicolous dwarf adders. Most members in the rupicolous clade are within a recent radiation *(*Fig. 1; *B. albanica*, *B. armata*, *B. cornuta*, *B. inornata* and *B. rubida*). *Bitis rubida* is paraphyletic with respect to *B. albanica* in the mitochondrial gene tree, and the occurrence of shared nuclear alleles is widespread among these five taxa (Fig. 2b). Monophyly of the remaining species within the rupicolous clade was supported across all gene trees. Notably a single *B. atropos* individual from Zimbabwe is divergent from South African individuals in the mitochondrial and UBN1 gene trees and also possesses unique alleles for both nuclear markers (Fig. 2b, Fig. S10 in Supporting Information).

Within the arenicolous *Calechidna* clade, the monophyly of *B. schneideri* was strongly supported across all analyses and it does not share any nuclear alleles with other species (Fig. 2b). The monophyly of *Bitis caudalis* was not supported in any of the analyses. Furthermore, the two polyphyletic mitochondrial lineages (*B. caudalis* L1 and L2) also failed to form a monophyletic group in the species and ML trees, with an alternative sister species relationship between *B. caudalis* L2 and *B. schneideri* being moderately supported (Fig. 1). This relationship was fully supported in the mitochondrial tree, with no nuclear allele sharing (Fig. 2). Further examination of the posterior sample of species trees showed that *B. caudalis* was paraphyletic in 98.9% of the posterior sample. The monophyly of *B. peringueyi* was supported in the mitochondrial and the ML trees, and this species shares nuclear alleles with *B. caudalis* L1 (Fig. 2b).

The dating analysis using a single Eurasian viperine fossil calibration provided a median estimated age for the basal divergence of *Bitis*, and the origin of the *Keniabitis* lineage, of 26.4 mya (95% credibility interval (CI) 20.7–33.7 mya). Divergence of the *B. arietans* lineage occurred 23.5 mya (95% CI 18.1–29.5 mya), and the *Macrocerastes* and *Calechidna* lineages separated 18.9 mya (95% CI 14.6–23.7 mya). The two *Calechidna* clades are estimated to have diverged 15.2 mya (95% CI 10.0–20.0 mya). Ancestors of the extant species within *Macrocerastes* and *Calechidna* are estimated to have arisen within approximately the last 10.5 my, with the most recent speciation events occurring in the *cornuta-inornata* (rupicolous) complex, which radiated within approximately the last 0.1–1.3 my.

The ancestral habitat state for the genus is unambiguously open habitat for both the APE and MESQUITE analyses. In addition, the estimated marginal ancestral states at each node were unequivocal with all proportional likelihood values > 0.98 (Fig. 1a, Fig. S12 in Supporting Information). There is a single transition to forest in the *Macrocerastes* clade,

with no transitions out of that habitat. The ancestral habitat reconstructions based on the ML topology produced essentially the same support values (> 0.98) for character states at each node (results not shown).

The ancestral area reconstruction with the DEC analysis suggests that *Bitis* originated in the Zambezian and Somalian/Ethiopian biogeographic regions (Fig. 3, Table S5 in Supporting Information). The divergence of *B. arietans* likely occurred in the Zambezian and Southern regions, with the divergence and diversification of the *Calechidna* clade accompanied by a transition into the Southern biogeographic region. The maximum likelihood topology differed from the species tree at the deepest node (placement of *B. arietans* and *B. worthingtoni*), resulting in the geographic origin of *Bitis* estimated as the Southern region with subsequent northward transition to the Zambezian region, followed later by a return transition to the Southern region (Fig. S13, Table S5 in Supporting Information). None of the analyses suggested a North African nor a Eurasian origin.

DISCUSSION

In Africa, groups that have undergone habitat-dependent evolution should show phylogenetic signatures that match the expansion of open habitats starting in the late Oligocene and the particularly notable habitat shifts in the Miocene. Our results show that the genus *Bitis* diverged from sister clades in the early Oligocene, and this does not seem to be in response to the reduction of ~~forests~~forest/woodland, given that most other African viper genera are also found in open habitat. Consistent with this, our analysis shows the ancestral state for *Bitis* as open habitat. Therefore, habitat-dependent evolution does not seem to be the initial driver of diversification within the African viperines, nor did it initiate the divergence of *Bitis* from other viperines. The majority of species level diversification within *Bitis* began in the late Miocene, with noteworthy divergence events occurring more recently for the species in hyper-arid regions. We found four well-supported clades that correspond to the currently recognised subgenera, and our phylogeny shows at least one cryptic taxon within *Bitis caudalis* and possibly *B. atropos*.

Is the evolution of *Bitis* habitat dependent?

We hypothesised that open habitat *Bitis* lineages (subgenera: *Calechidna*, *Keniabitis* and *Bitis*) diverged either in response to the initial but gradual aridification of Africa (Eocene/Oligocene), or later during the rapid mid-Miocene expansion of open habitats. The ancestral state for the genus is an open habitat at the basal node (median estimated age 26.2 mya, 95% credibility interval 20.6–33.7 mya), with one shift to forest by *Macrocerastes* in the mid-Miocene. Given that the ancestral state is open habitat, the origin of *Bitis* does not appear to be a case of habitat-dependent evolution in response to a shift from closed to open habitats, because the genus emerged at a time when open habitats already existed. Indeed, it is likely that closed or dense canopy forest and woodland formed a mosaic with open habitats (Linder, 2017) providing ample opportunity for diversification into open vegetation. The 'forest-living' ancestral condition for the entire subfamily is itself questionable, as most other viperine lineages except *Atheris* and some *Causus* inhabit primarily open formations. It is highly likely then, that Viperinae evolved in an open habitat setting in the Oligocene, with multiple shifts into forest by certain lineages (i.e. *Atheris* and subgenus *Macrocerastes*).

Although the origin of *Bitis* in the Oligocene is inconsistent with habitat-dependent evolution, within the genus there are indications of habitat-dependent diversification. Vicariance initiated by the fragmentation of forest during the late-Miocene and Pliocene may have contributed to cladogenesis within the forest-dwelling *Macrocerastes*. Furthermore, the mid-Miocene divergence of the *Calechidna* clade coincides with the intensification of the Benguela oceanic current and associated development of the arid conditions in the west, including establishment of the Namib Desert (Scott & Anderson, 1997; Udeze & Oboh-Ikuenobe, 2005). All four arenicolous *Calechidna* lineages occur in the west, suggesting they shifted to the arid niche as it became available. Diversification within *Calechidna* is more recent, within the last ca. 5 mya. This corresponds well to the late Miocene/Pliocene shift from moist woodland and forest to the present day arid open habitat conditions in Namaqualand and the Karoo (Scott & Anderson, 1997; see Fig. S14 in Supporting Information for these localities). It is likely that an arid-living ancestral clade from the Namib region (*B. peringueyi* and *B. caudalis* L1) diversified and shifted to the more southern central Karoo (*B. caudalis* L2) and west coast Namaqualand (*B. schneideri*) as habitat became more xeric. However, throughout the Pleistocene the climate varied widely due to glacial cycling. Indeed, the central Karoo is considered to have high 'climate velocity', whereby the biome has shifted in position and extent during the Pleistocene (Tolley *et al*., 2014). The current biomes have apparently been relatively stable in extent through the Holocene (Scott & Anderson, 1997). Although the region has been climatically dynamic, there has been a long-term aridification trend which has undoubtedly influenced cladogenesis within the *Calechidna*. The formation of the arid west and Namib Desert has also been linked to evolutionary diversification in lizards (Lamb & Bauer, 2003, 2006; Makokha *et al*., 2007; Edwards *et al*., 2016), and this extreme environment certainly must have played a role in speciation and adaptation of arid-living fauna.

In addition to habitat factors, divergence timings within *Bitis* also correspond with geological events. Specifically, the divergence of *B. parviocula* from its sister clade coincides with the ~~onset~~extension of the Main Ethiopian rift which began around 11 mya (postdating the initial rifting of the Red Sea/Gulf of Aden in ~~Ethiopia 11 mya (~~the late Oligocene; Wolfenden *et al*., 2004). Considering the limited distribution of *B. parviocula* along the Ethiopian Rift, this result strongly suggests a causal role for these geological processes in the origin of this species, as has been suggested for other East African squamate lineages (Matthee *et al*., 2004; Wüster *et al*., 2007; Tolley *et al*., 2011). It should be noted that genetic data for the newly described *B. harenna* is still lacking, but is essential to test this hypothesis. In contrast, however, *B. worthingtoni* currently has a limited distribution along the Kenyan Rift Valley but divergence from its sister clade considerably pre-dates the onset of rift formation and volcanism in Kenya, 16–20 mya (Chorowicz, 2005), suggesting that these geological events were not involved in the divergence of this taxon.

We acknowledge that our dating analysis was calibrated using a single Eurasian viper fossil, so our interpretations regarding timing of events should be treated with some caution. However, other molecular phylogenies that include vipers also place the divergence of *Bitis* from other vipers within the Oligocene (ranging between 35–40 mya; Wüster *et al*., 2008, Alencar *et al*., 2016), corresponding with our own analysis that suggests a divergence around 31.9 mya (95% CI 26–40 mya). Inclusion of additional calibration points may refine

the diversification dates within *Bitis*, but it is unlikely that the dating would shift so substantially as to alter our main interpretations.

The geographic origin of *Bitis* unfortunately remains elusive, in part due to the differing topologies for the species tree and the maximum likelihood tree at the deepest node. The species tree analysis showed a Zambezian+Ethiopian/Somalian ancestral area, whereas the ML topology suggests a southern African origin. The analysis would likely be improved with the addition of missing genera (*Atheris*, *Eristicophis*, *Macrovipera*, *Montatheris*, *Proatheris*, *Pseudocerastes*) and species (*B. heraldica, B. harenna*). The Zambezian and North African regions experienced substantial reduction in forest (opening of habitat) during the Oligocene (Morley, 2007). Both analyses are in agreement that the genus did not originate in North Africa, but rather in the south/eastern region of the continent, with the Zambezian region playing an important role. Therefore, we suggest that the opening of habitat in the Zambezian region initiated the diversification of this genus. It also appears that the common ancestor for the crown groups occurred in the Zambezian region (ca. 20–25 mya), and then split into a southern African clade (*Calechidna*) and a more widespread clade centred in the eastern-central portion of the continent (*Macrocerastes*).

Phylogeny and systematics of *Bitis*

Our results provide new information on the phylogeny and systematics of *Bitis.* A key question which has remained equivocal despite numerous phylogenetic studies is relationships among the *Bitis* subgenera, specifically the relative positions of *Keniabitis* and the *B. arietans* lineage (Lenk *et al.,* 1999; Wüster *et al*., 2008, Alencar *et al.,* 2016). Through multispecies coalescent analysis of mitochondrial and three nuclear loci we were able to resolve this relationship with high posterior support, placing *B. arietans* as sister to *Macrocerastes* and *Calechidna*, with *Keniabitis* in turn sister to this clade. Achieving this robust phylogenetic hypothesis for *Bitis* subgenera will benefit future studies on the evolution and diversification of this group.

Furthermore, we suggest that current taxonomy may not fully capture species diversity within the subgenus *Calechidna*. The four samples of *B. caudalis* analysed comprise two divergent and polyphyletic mitochondrial lineages. Multispecies coalescent analysis of these lineages suggests that *B. caudalis* L2 and *B. schneideri* (both from southwestern South Africa) share a recent common ancestry, whereas *B. caudalis* L1 and *B. peringueyi* (both from western Namibia) (Fig. S15 in Supporting Information) share a recent common ancestry. The maximum likelihood analysis however, differed for these relationships although each of these clades was still supported as distinct. *Bitis caudalis* is widespread across south-western Africa, occurring from southern Angola southwards to the Western Cape Province of South Africa, and eastwards to southern Zimbabwe. Because our sampling was limited, we cannot make firm conclusions regarding these relationships. Indeed, a comprehensive phylogeographic analysis of this widespread taxon is a priority for future studies on *Bitis*, particularly as the two analyses showed slightly different relationships between the clades.

Further indication of potentially cryptic species diversity was found among *B. atropos* populations. Specifically, the Zimbabwean *B. atropos* possessed unique alleles for two

nuclear markers (Fig. 2b), and exhibited significant levels of mitochondrial divergence from conspecific samples (all from the Western Cape, South Africa), comparable with divergences of other interspecific rather than intraspecific relationships within *Calechidna* (Fig. 2a). *Bitis atropos* has a fragmented distribution with populations occurring along the Cape Fold Mountains in the Western and Eastern Cape Provinces of South Africa, and additional allopatric populations in the KwaZulu-Natal and Mpumalanga provinces of South Africa, and in Zimbabwe. It was hypothesised that these isolated populations represent a assemblage of sibling species (Branch, 1999). It was later shown that the *B. atropos* 'complex' comprises a suite of cryptic species that apparently originated in stepwise fashion from north to south, associated with isolation of montane grassland habitats of the Great Escarpment (Kelly *et al.,* 2011). Together with our results, this highlights *B. atropos* as an important focus for future research efforts.

The *cornuta-inornata* complex comprises five morphologically and ecologically differentiated species (Branch, 1999), which our molecular dating analysis shows to have radiated much more recently than other *Bitis* clades (within the last ca. 1.2 my). Analysis of mitochondrial sequences and the maximum likelihood analysis recovered *B. albanica* and *B. rubida* as polyphyletic, and these together showed little differentiation from *B. inornata*. Sharing of nuclear alleles was also evident among these three taxa as well as among the other species in the complex, *B. armata* and *B. cornuta*. These genetic patterns are consistent with a recent radiation of these species, and any taxonomic interpretations based on our limited sampling would be premature. The relationships between these taxa might become better understood with denser sampling of individuals and additional genetic loci.

Above the species-level, previous discussions of *Bitis* systematics have considered their higher level taxonomy, specifically whether the four subgeneric clades may warrant elevation to genus level (Herrmann & Joger, 1997; Lenk *et al*., 1999). Changes in nomenclature are justified in cases where current taxonomy does not adequately portray evolutionary relationships, but this must be balanced against the potential negative impacts of taxonomic changes on the wider scientific community. Given the strong support for monophyly of the genus *Bitis* as currently defined, we share the view of Wüster *et al.* (2008) that splitting of this historically stable group would only serve to confuse the nomenclature and hinder information retrieval without significantly enhancing our understanding of the evolutionary history of the genus. The continued recognition of the *Bitis* subgenera, however, does provide an effective way of highlighting the major evolutionary and ecological divisions within the genus whilst avoiding any potentially negative effects of generic reassignment. Overall, this results in a more information-rich classification (Wallach *et al*., 2009).

CONCLUSION

~~We provide evidence of the evolutionary radiation of open habitat lineages prior to the major expansion of these habitats in the mid-Miocene.~~Our analysis was limited to a dichotomy of open/closed habitats, yet the vegetation of Africa was surely more complex through space and time. Therefore, we are limited to interpretations relating only to broad scale patterns; yet diversification within *Bitis*, and indeed within viperines, could easily have been driven by nuances rather than the generalities that are characterise our study. Until such time that the complexities of African paleo-vegetation are revealed, broad patterns

over large time scales will characterise our best knowledge. Overall, we show that the diversification of *Bitis* likely began in open habitats in the late Oligocene/early Miocene, prior to the major expansion of such habitats in the mid-Miocene. This contrasts strongly with open habitat mammalian lineages which are shown by the fossil record to have diversified much later, following the expansion of C_4 grasslands in the late Pliocene and Pleistocene (Vrba, 1992; Wesselman, 1985; Bobe *et al*., 2002; Bobe & Behrensmeyer, 2004). Overall, our results highlight the need for taxonomic breadth in achieving a holistic understanding of faunal evolution in Africa~~.~~, as well as for fine-scale analyses that aim to incorporate subtleties of vegetation and climatic dynamics.

Table 1. Taxonomy of *Bitis* and habitat preference for each species.

Subgenus	**species**		**habitat**
Macrocerastes	*B. gabonica*	East African Gaboon Adder	Tropical and montane forest
	B. rhinoceros	West African Gaboon Adder	
	B. nasicornis	Rhinoceros Viper	
	B. parviocula	Ethiopian Mountain Adder	
	*B. harenna**	Bale Mountains Adder	
Calechidna	*B. albanica*	Albany Adder	lowland and montane rocky or gravely grassland, karroid and Sclerophyllous scrub
	B. armata	Southern Adder	
	B. atropos	Berg Adder	
	B. cornuta	Many-horned Adder	
	*B. heraldica**	Angolan Adder	
	B. inornata	Plain Mountain Adder	
	B. rubida	Red Adder	
	B. xeropaga	Desert Mountain Adder	
	B. caudalis Lineage 1	Horned Adder	sandy savanna & karroid scrub and alluvial soils
	B. caudalis Lineage 2		
	B. peringueyi	Peringuey's Adder	Namib sand sea
	B. schneideri	Namaqua Dwarf Adder	coastal sand dunes
Bitis (type subgenus)	*B. arietans* complex	Puff Adder	open savanna, grassland and karroid scrub absent from forest and desert
Keniabitis	*B. worthingtoni*	Kenya Horned Viper	montane grassland and scrub

*Subgeneric assignment not confirmed by genetic analysis

Figure 1 a) *Bitis* MSC species tree. Nodes are centred on the median age from the posterior sample, and the 95% CIs indicated by the blue bars. Node support values are Bayesian posterior clade probabilities. Support values are from the three locus analysis (those preceded by asterisks were supported in the four locus analysis). The major subgeneric *Bitis* clades are indicated to the right of the figure and are coloured according to habitat preference. The general shift from forest (green) to open (yellow) habitats in the mid-Miocene is indicated, with inset maps showing ~~general~~rough extent of forest ~~(~~/woodland mosaic (stippled green) in the Oligocene and at present (blue indicates areas inundated by sea). The ancestral character states at major nodes are shown by coloured circles. b) Maximum likelihood bootstrap consensus tree for the concatenated four gene analysis, with terminal tips collapsed for each clade/species. Bootstrap values are given for nodes with > 70% support. The topology differs from the species tree at the nodes indicated by arrows. For both figures, outgroup taxa have been removed for clarity but are shown in Supporting information.

Figure 2 a) Mitochondrial gene tree estimated in the three-locus MSC analysis for *Bitis*. Filled circles at nodes indicate Bayesian clade support of 1.0, whereas values < 1.0 are given numerically. b) Matrix of *Bitis* species showing instances of shared alleles (filled squares) for the nuclear PRLR (below the diagonal) and UBN1 (above the diagonal) genes. Asterisks indicate species for which monophyly was supported by posterior probabilities ≥ 0.9 in the nuclear gene trees estimated in the three-locus MSC analysis for PRLR (vertical list, see Fig. S4 in Supporting Information) and UBN1 (horizontal list, refer to Fig. S5 in Supporting Information).

Figure 3 Ancestral area reconstruction for *Bitis*. Proportional likelihood values are shown for each node by coloured doughnut charts (colour codes match key). Area coding for each taxon/tip is indicated: A-Eurasia, B-North Africa, C-Congolian, D-Ethiopian/Somalian, E-Sudanian, F-Zambezian, G-Southern, and corresponds to the map of biogeographic regions for Africa (inset).

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Biosketches

Axel Barlow is interested in studying the evolutionary history of populations using DNA sequence data. His work encompasses a range of vertebrate taxa across a variety of geographic regions and temporal scales. He is also interested in the development of new laboratory and analytical approaches that can be applied to evolutionary questions.

Wolfgang Wüster is a herpetologist interested in the systematics, phylogeography and phylogeny of venomous snakes and the evolution of snake venoms.

Krystal A. Tolley is interested in understanding the historical processes that generate patterns of diversity in African reptiles using biogeographic and phylogenetic approaches.

Author Contributions A.B. and W.W. funded and designed the project. A.B. and C.M.R.K. carried out laboratory work. A.B., W.W. and K.A.T. analysed the data, interpreted the results and wrote the manuscript. All authors contributed to sampling and to the manuscript text.

Supporting Figures[1]

Figure S1. Paleo-vegetation in African during the Oligocene based on Morley 2007
Figure S2. Biogeographic regions of Africa based on Linder et al. 2012
Figure S3. Maximum likelihood tree for phased PRLR dataset
Figure S4. Maximum likelihood tree for unphased PRLR dataset
Figure S5. Maximum likelihood tree for phased UBN1 dataset
Figure S6. Maximum likelihood tree for unphased UBN1 dataset
Figure S7. MSC species tree from the three locus analysis (two linked mitochondrial, two unlinked nuclear)
Figure S8. Maximum likelihood tree for three locus analysis (two linked mitochondrial, two unlinked unphased nuclear)
Figure S9. MSC PRLR gene tree from the three locus analysis
Figure S10. MSC UBN1 gene tree from the three locus analysis
Figure S11. Subgenus level MSC species tree from the four locus analysis
Figure S12. Character state coding and results of ancestral characters state optimisation for habitat type

Figure S13. Character state coding and results of ancestral characters state optimisation for area using the maximum likelihood phylogeny
Figure S14. Map of southern Africa with place names indicated
Figure S15. Interpreted distributions of *Bitis caudalis*, *B. peringueyi* and *B. schneideri*

[1]Figures S3-S11 are available as nexus files on Dryad, which can be visualised interactively using FigTree or similar software.

Supporting Tables
Table S1. Details of individuals sequenced for this study with corresponding GenBank accession numbers
Table S2. Ancestral area coding
Table S3. Ancestral range constraints
Table S4. Dispersal probabilities between regions at four time periods
Table S5. Proportional likelihoods for ancestral area reconstructions using a) the species tree, b) the maximum likelihood tree

Supporting data deposited in Dryad

mtDNA alignment as a nexus file in Dryad

PRLR alignment as a nexus file in Dryad

UBN1 alignment as a nexus file in Dryad

Responses to Editor and Reviewers comments
Barlow et al. Ancient habitat shifts and organismal diversification are decoupled in the African viper genus *Bitis* (Serpentes: Viperidae)

FURTHER COMMENTS FROM THE CHIEF EDITOR
This is a nice paper!

- *Thanks!*

EDITOR'S COMMENTS TO AUTHOR
Editor: Procheş, Şerban
If the technical comments from Reviewer 2 are answered convincingly, re-review should not be necessary.

Also attend to all other comments and suggested corrections from both reviewers and myself.
Namib Deserts in Fig S7 should be capital D - but I'm not sure the figure is strictly necessary.

SP comment in document: I'm wondering whether it would not make sense to have green under the branches in Macrocerastes, or to have no colour at all under the branches, just a narrow gradient at the bottom indicating overall climatic trends?

- *I see what you mean. I gave some thought on how to do this without making the figure confusing, and I couldn't come up with a good solution. So I took the easy way out and reorganised the figure legend a bit and added an explanatory line: "*The general shift from forest (green) to open (yellow) habitats in the mid-Miocene is indicated, although *Macrocerastes* persisted in forest patches throughout the Miocene to the present". *The ancestral character states at major nodes shown by coloured circles."*

- *All other comments in pdf document from SP addressed.*

Referee: 1
Comments to the Author
This is a welldone work that deserves to be published without much change. Yet it does not present surprising results.

The phylogeny shown was expected by previous works that is cited. Especially the driving hypothesis should not be that forest living is the primitive condition (which is suggested in the paper), but all previous evidence already suggested that Bitis originated in open country. This is merely confirmed by this analysis, but of course in an good and well documented way.

- *This is not entirely accurate. There is one Bitis phylogeny to date that used genetic data (all from genbank) and morphological data combined in an analysis and who included essentially one representative(from genbank) per species (Wittenberg et al. 2014). That study also had several missing taxa, which we have now included (B. inornata, B. albanica) and our better geographic sampling for most taxa does in fact show new results, specifically that B. caudalis is not monophyletic. (The two caudalis clades each being related to schneideri and peringueyi, respectively, rather than each other.) In addition, our phylogeny is dated whereas Wittenberg et al is not, which allows us to delve into the ancestral habitat reconstructions in a temporal context.*

- *Furthermore, the Wittenberg study uses fairly sparse genbank data with little overlapping gene regions between taxa. Their Table S1 is pasted in below.*

- *None of the other higher level (dated) viper phylogenies to date have looked at Bitis to this level of detail. Our phylogeny includes: more taxa, is dated, has improved geographic coverage, is multilocus, and uses a dataset with overlapping gene regions between taxa.*

- *Of the various higher level viper phylogenies, none have stated an open habitat origin hypothesis. The Wittenberg et al. publication indicates only that "…a viper preferring open habitat*
- *could have become widespread before giving rise to other forms" however, they do not specifically pose the hypothesis nor do they test it. In fact, because their phylogeny is not dated, they would not have any way to make the sorts of inferences that we make.*

- *from Wittenberg et al:*
- Table S1. Taxa and data used in DNA analysis. Abbreviations are as follows: ND4 = NADH dehydrogenase subunit 4, Cyt b = cytochrome b, 16S and 12S = small ribosomal RNA fragments, ND2 = NADH dehydrogenase subunit 2, PRLR = prolactin receptor, Ba34 = anonymous nuclear locus from Barlow et al. (2012).

Species	Voucher	Locality	ND4	Cyt *b*	16S	12S	ND2	PRLR	Ba34
B. arietans	WW 1571	Morocco	JX114182	JX114012			JX073288	JX073299	JX073330
	T. Mazuch, private collection	Agadir, Morocco			EU624280	EU624245			
B. armata	WW 1729	South Africa					JX073291	JX073302	JX073324
B. atropos	WW 1446	Bettys Bay, Western Cape, South Africa	EU624214		EU624281	EU624246			
	WW 1445	South Africa					JX073287	JX073298	JX073325
	PEM, no number	Swartburg, South Africa		AJ275691					
B. caudalis	WW 1555	Springbok, Northern Cape, South Africa	EU624215		EU624282	EU624247			
	WW 2445	South Africa					JX073293	JX073304	JX073322
	ZMFK 65212	Swakopmund, Namibia		AJ275693					
B. cornuta	WW 1554	Near Springbok, Northern Cape, South Africa	EU624216			EU624248			
	WW 1589	Near Springbok, Northern Cape, South Africa		EU624305	EU624283				
B. gabonica	WW 1330	St. Lucia, LwaZulu Natal, South Africa	EU624217		EU624284	EU624249			
	WW 2714	Democratic Republic of the Congo					JX073296	JX073307	JX073328

	ZMFK 64335	Kivu, Democratic Republic of the Congo		AJ275695					
B. nasicornis	CAS 207874	Bioko, Equatorial Guinea	DQ305475	DQ305457	DQ305434	DQ305411			
B. parviocula	WW 2980	Ethiopia					JX073292	JX073303	JX073327
B. peringueyi	CAS 193863	Swakopmund, Namibia	DQ305476	DQ305458	DQ305435	DQ305412			
B. rhinoceros	WW 1287, Liverpool School of Tropical Medicine live coll.	Ghana	EU624218		EU624285	EU624250			
	HLMD RA-2909	Togo		AJ275696					
B. rubida	WW 1397	80 km N Ceres, South Africa	EU624219	EU624306	EU624286	EU624251			
	WW 1712	South Africa					JX073290	JX073301	JX073323
B. schneideri	WW 2811	South Africa					JX073297	JX073308	JX073321
B. worthingtoni	WW 1369	Kenya	EU624220			EU624252			
	WW 2625	Kenya					JX073295	JX073306	JX073331
	NHMN, no number	Kenya		AJ275692	AJ275745				
B. xeropaga	WW 1380	unknown	EU624221	EU624307	EU624287	EU624253			
	WW 2621	South Africa					JX073294	JX073305	JX073326
Vipera berus	WW 199	United Kingdom	EU624233			EU624267			
	–	France		AY321091			AY321075		
	HLMD RA-1665	Göteborg, Sweden			AJ275772				

I only found a few corrections to be done:
- Line 58 (abstract): Keniabitis.
- Line 84: 2014, not 2104.
- Line 342: If Calechidna is composed of 2 clades, B.xeropaga and B.atropos must be included (not listed here).
- Line 369: "Extant species ... are estimated to have arisen..." - The extant species are certainly not so old. Netter
write: "Predecessors of extant species have arisen".
- Line 424:habitat became

- *All done.*
- *Regarding L342: changed to "*Most members in the rupicolous clade are within a recent radiation *" but the species list was kept the same. This is because the paragraph is about those particular species (not about B. xeropaga and B. atropos).*

Referee: 2

Comments to the Author
This is well-written paper about an interesting radiation of open-habitat associated snakes in the genus Bitis. The paper is mostly easy to follow and as questions were flagged, the next paragraph answered then, which was pleasing. My comments are mostly minor and I hope the authors find them useful in revising their manuscript.

Introduction
Lines 98-103. My single major comment pertains to what the authors call the "habitat-specific hypothesis". Even by the end of the discussion, I did not fully understand what the authors are getting at with this, as it is poorly defined in the introduction. Why is this not just ecological speciation, a process well-documented in the literature. You could argue that a specific time dimension is added along the axis of habitat availability through time, but this is just the shrinking and expanding of biomes, and not the specific underlying process, which is speciation across ecological barriers (habitats, ecotones, gradients, biomes – whatever you want to call it).

- *Yes, it can be basically considered ecological speciation. The nuance is that we are pinpointing the 'ecological' part of it, that is, what exactly about the environment is driving this (open vs closed habitat). Text modified and references added:*
- *"…This paradigm essentially points to ecological speciation where diversification is driven by divergent selection in different environments (e.g. Rundil & Schluter, 2005; Schluter, 2009). Here, we adopt the term habitat-dependent evolution to specific refer to ecological diversification for lineages inhabiting novel habitats in response to reorganisation of habitat types on the African continent."*

Table 1. Could a column with common names be added?
- *Done.*

Methods
Appendix S1 appears to be missing nearly all the GenBank sequence numbers, these should be added.
- *Yes we know they are missing because the draft submitted often is done without these numbers, which are provided within this revision. We should have stated that in the original cover letter.*

I could see no dashes on the PDF version downloaded. What are the voucher numbers – these look like personal collector numbers? If the later another column detailing the institution that has or will accession the sample/specimen should be added. A column should also be added to indicate the type of sample, blood, specimen etc. It is critical that the GenBank numbers can be connected with the individuals that are represented by actual specimens, which will be of fundamental importance if there are cryptic species as suggested in the present study.

- *The samples were tissues only, as the individuals were released or in live collections. This has been made clearer in the Methods.*
- *"All individuals were released after sampling."*

Data – Lines 217-218. It concerns me that for 16s and some nuclear loci only the forward strand was sequenced. Data is usually much more reliable if both strands are sequenced as discrepancies are easily identified. Can a sentence be added about quality controls at this step?

- *Of course, it would have been very nice to have sequenced everything in both directions, but we had a strict funding limitation that did not allow for this. That said, we believe that the*

number of potential false base calls for 16S (or other genes) are few and would not provide a false signal that would result in a different outcome in our phylogeny. Regardless, we are aware that mis-called bases is an issue that is better to not have, if possible. So we checked everything carefully in CodonCode Aligner, only used clean sequences, re-sequenced any sequence with questionable stretches, and inserted Ns in case of persisting doubt. A sentence to this effect has been added to the Methods, around line 220.

Lines 222-225. Given that parts of 16s were omitted and 30 bp was omitted from UBN1 could these alignments be provided and the omitted regions highlighted. Without these data the analyses are not repeatable.

- *If accepted, we will submit the alignments as supplemental information to Dryad in the form of nexus files. We did not do this for the first submission, because the JBI system would not take the files.*

Lines 229-231. What threshold was used to determine that a SNP was phased with confidence? In many instances this can be challenging. Did you use the output of PHASE as is or was for instance, a PP=0.70 or 0.95 used as the basis for deciding if a SNP was successfully phased? How were SNPs coded that did not meet this threshold, for example those with small minor allele frequencies? These aspects of the data need to be clarified.

- The phase analysis was carried out exactly as described in the methods: in DNAsp using default parameters. Thus the information is sufficient for this analysis to be replicated exactly. Regarding the reliability of this particular implementation of the PHASE method, we decided the best approach in this case was to investigate the extent to which the phasing procedure may introduce measurable changes in phylogenetic signal. We calculated and compared maximum likelihood trees for each nuclear locus generated from both the phased and unphased alignments. This showed exeptional conservation of relationhips and bootstrap values for all nodes above the species level between the phased and unphased treatments. Thus, the phase analysis as implemented here is reliable and has not distorted phylogenetic signals in any obvious or measurable way. This new analysis is reported in the methods section and the trees are included in the supplementary materials

Phylogenetic analyses. I would like to see maximum likelihood analyses performed to complement the Bayesian analyses. Occasionally, Bayesian analyses can be misleading, particularly when performed in BEAST, which has some significant biases. These biases may be exacerbated when species-tree and dating are performed at the same time. The ML support values could be added to Figures 1 and 2, and if the topologies are different, the ML topologies could be placed in the supplementary docs.

- *A ML analysis has been added for the mt only, 4 gene phased dataset, and the 4 gene unphased dataset, to address the comment above as well. The results show a slightly different topology and this has been added to Figure 1. We have also pointed out the differences and discussed them. The knock on effect was that we also had to run extra ancestral reconstructions to see if the topology differences would affect the outcome. This has all been added to the Methods/Results/Discussion.*

Results
To more fully explore this dataset could an ancestral area reconstruction be conducted for the genus? This aspect of the paper is presently understated and could add quite a lot given that this

paper is targeted at J. of Biogeography. How many of the potential outgroup lineages were sampled in Africa? The authors partly address this, but I want some greater assurance that it is not simply the choice of outgroup species that is driving the ancestral area reconstruction.

- *This comment is a bit confusing. I think it is probably two separate questions and will treat it as such.*

- *First part of question:*
- *Yes an ancestral area reconstruction can be done, although this is was not intended to be part of the study. Although we have now added it, that addition has knock on effects because the Intro and Discussion needed altering as well in order to place this analysis in context.*

- *In addition we have to treat it with caution for the same reasons brought up by the reviewer, that there are some genera missing. In particular, there are two African genera missing from the outgroup (Atheris and Proatheris) from East Africa and these might be sister to Bitis, so we would really want them in the analysis. However, the results of the current analysis suggest an East/southern African origin anyway, so it's not inconsistent with what we would expect if Atheris and Proatheris were included. Their inclusion would probably just increase the confidence in the result.*

- *2nd part of question:*
- *For the outgroup, six other genera in the Viperinae were included (leaving 5 missing genera). There was a fair spread of African and European genera included (with 1-3 species to represent each). Some additional clarification on this now has been added to the Methods.*

- *I do not understand the latter part of this comment ('choice of outgroup species that is driving the ancestral area reconstruction') because an ancestral area reconstruction was not done in the first version. Perhaps the reviewer means – the ancestral habitat reconstruction?*

- *Yes missing taxa could affect this, but of the five missing genera, all but one would be considered open habitat living. So it is fairly unlikely that inclusion of the missing genera would have made a difference in the general interpretation. Their inclusion might have increased confidence, but probably not changed the outcome. Some clarification along these lines has been added to the ancestral habitat reconstruction Methods. "Because five Viperinae genera were missing from our analysis, we must treat the results of this analysis with caution. However, it should be noted that four of these five missing genera occur in open habitat, with only Atheris found in forest. Whether inclusion of Atheris and the other genera would change the results would require a more comprehensive Viperinae phylogeny."*

Discussion
Lines 467-474. Could some text be added to provide greater detail of the present known geographical circumscription of B. caudalis and each of the two lineages identified from the sequence data. A map, even in the supplementary documents, may be a useful aid to interpretation.

- *The text was clarified: "Multispecies coalescent analysis of these lineages suggests that B. caudalis L2 and B. schneideri both from southwestern South Africa, share a recent common ancestry, whereas B. caudalis L1 and B. peringueyi, both from western Namibia, share a recent common ancestry (Fig. S8 in Supporting Information)"*

- *A map has been added to the supporting info.*

Sub-genetic names. I realize this is the authors' prerogative, but it makes no sense to me to use sub-generic names, especially when in the text these names are used in the same fashion as genera. To me the use of sub-genera just adds more confusion and so should be avoided.

- *We decided to include the sub-genera names to avoid any need (or perhaps to pre-empt the suggestion) that the genus be split into multiple genera. We strongly advocate keeping a single genus, for a number of reasons: (i) The use of subgenera adds an additional tier of phylogenetic information to the nomenclature, whereas splitting the genus would simply refocus the plane of phylogenetic resolution from broader to finer-scale relationships; (ii) this is an iconic genus in African herpetology, with a very large body of both scientific and popular literature on the different species; splitting it would cause considerable confusion and greatly hinder information retrieval . Thus, to avoid destabilising the taxonomy unnecessarily while maximising phylogenetic resolution, it seemed better to keep the genus as one, but using the sub-genera does allow for recognising that there are some very distinct clades there.*